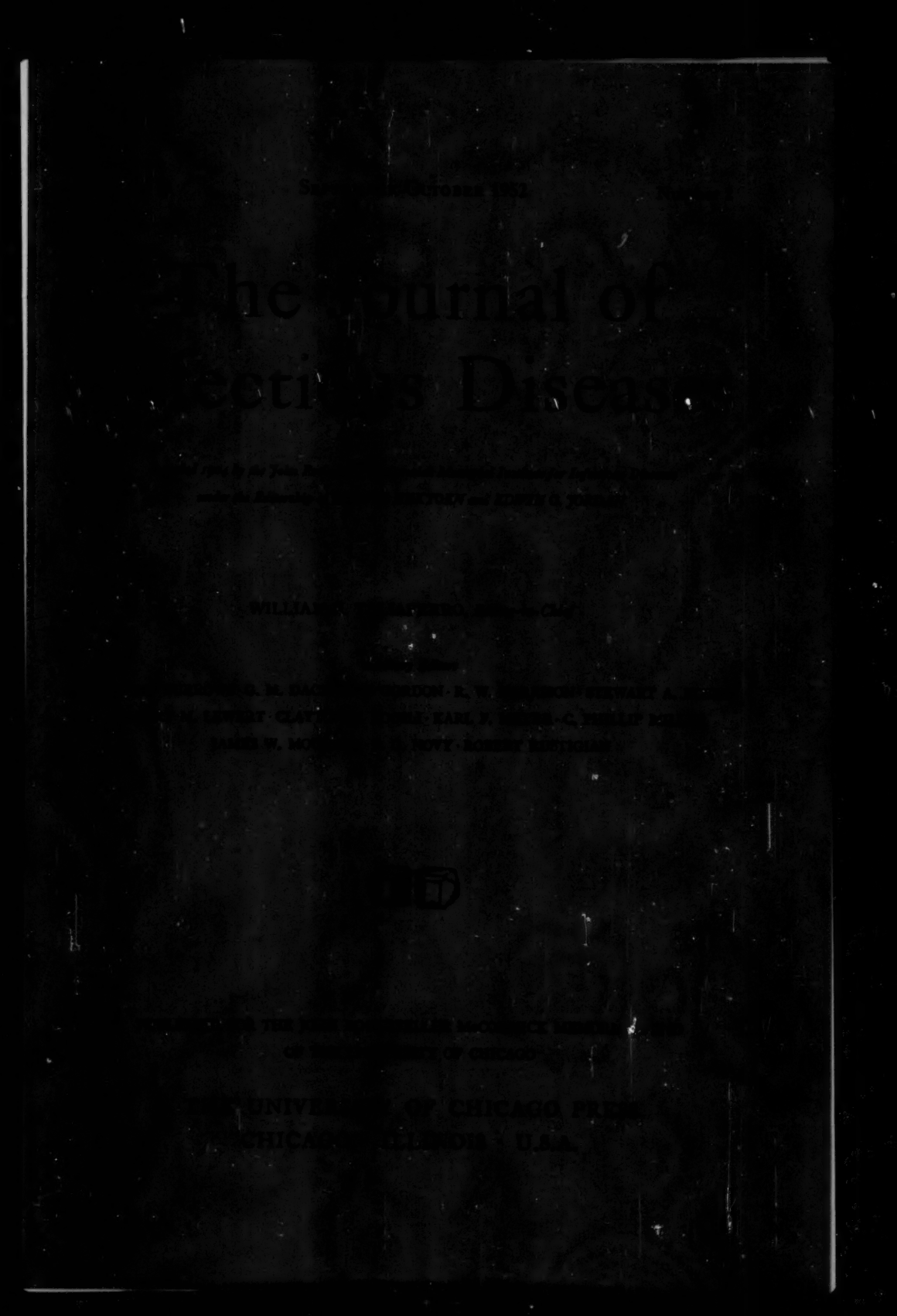

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THE LOCALIZATION OF X-RAY INJURY TO THE INITIAL PHASES OF ANTIBODY RESPONSE

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The importance of the time of immunization with respect to irradiation has been widely recognized by previous investigators studying the effect of X rays on antibody formation. As early as 1908, Benjamin and Sluka found that the precipitin titer was lower and antigen disappeared more slowly in rabbits injected with bovine serum just after several doses of X rays than in rabbits injected with the same amount of antigen 4 days before irradiation or in nonirradiated controls. This time relationship between immunization and the inhibitory effect of X irradiation has been repeatedly corroborated as pointed out in a recent review by the senior authors (1951). The general agreement of authors that the immune response is highly resistant to X irradiation after the antigen has been in the body for only a short time indicates that X-ray damage is largely limited to the first part of the immune process.

The present investigation was undertaken to define more exactly the relationship between the time of X irradiation and the time of antigen injection and to characterize more fully X-ray damage to antibody production. It was carried out on antisheep hemolysins in rabbits, and antibody production was in general characterized in terms of the length of the induction period, the peak serum antibody titer and the rate of antibody accumulation. We (1952 and 1952b) have published two preliminary reports on certain phases of the work. In a large measure, the data corroborate the findings of Dixon, Talmage and Bukantz, whose preliminary report (1951) appeared while our work was in progress, and Dixon, Talmage and Maurer (1952), who kindly supplied us with a manuscript of their final paper while it was still in press. Their work and other pertinent studies will be considered in detail in the discussion.

MATERIALS AND METHODS

The same materials and methods were used as previously described (W. H. and L. G. Taliaferro, 1950, 1950b, 1951b and 1952). Only a few salient details and modifications in procedure will be outlined here. The antigen consisted exclusively of sheep red cells from one sheep. Suspensions of red cells, whether used for injecting or testing, were standardized in terms of hemoglobin so that 1 ml of the suspension plus 4 ml distilled water gave a reading of 150 on the Klett-Summerson photoelectric colorimeter with a 54 filter (approximately 500 to 570 mμ). In the present study, 1.25 ml of this standardized suspension per kg body weight was injected into the majority of the rabbits. For convenience, it may be briefly designated as 1.25 ml of a 1% suspension or 2×10^8 cells. In our previous papers this amount was called the intermediate dose. It was injected into the left ear vein. Blood from the rabbits for the titration of hemolysin was usually obtained

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The authors wish to thank Dr. Julius M. Coon, Director of the U. S. Air Force Radiation Laboratory at the University of Chicago, for the use of irradiation facilities; Dr. Austin M. Brues and Mr. Joseph E. Trier of the Argonne National Laboratory for irradiating some of the animals; and Mr. Francis Murphy, Miss Gloria Goicochea and Mr. Edward Neal for technical assistance.

daily for a week or 10 days after the initial injection and 3 or 4 times a week for the following 2 or 3 weeks. Sometimes bleedings were continued longer—occasionally for another month.

All hemolysin titers are given in 50% hemolytic units. The unit may be defined as the amount of serum which produces 50% hemolysis of 2 ml of the afore-mentioned photometrically standardized suspension of red cells (approximately a 1% suspension) in the presence of four 50% units of complement. Titers were read from a plot, according to the von Krogh equation, of the amount of serum against the degree of hemolysis determined photometrically. In the semilogarithmic graphs such titers are expressed directly in ml of serum. In the text and figure 2 they are expressed as the number of 50% hemolytic units per ml, which is identical to the reciprocal of the titer given in the semilogarithmic graphs.

The data given in the tables and figures indicate the length of the induction period, and the amount and rate of antibody accumulation in the serum to peak titer. In the earlier work by the senior authors (1950, 1951b and 1952), it was found that titers in a given rabbit rise and fall according to a series of discontinuous semilogarithmic curves described by the first order equation

$$(1) \qquad A_t = A_0 e^{kt}$$

in which A_t and A_0 represent the antibody titers at times t and 0, respectively, and k represents the constant of increment. From such curves were obtained fitted titers, including peak titer, the length of the induction period and initial rise, and total time to peak titer. Such values are used for all means, etc. They are only slightly different but are probably more reliable than actual values. Declines in hemolysin titer are not only indicated by rates, as obtained in equation 1, but by the apparent half life

$$t_{1/2} = \frac{0.693}{k}.$$

Peak titers and rates of the secondary antibody response are derived as outlined by the senior authors (1952). Such values represent an approximation of the new antibody accumulated in the serum during the secondary responses. They were obtained graphically by subtracting the amount of residual antibody remaining from the initial response from the actual observed peak titer after the second antigen injection. The residual antibody from the first response was assumed to be the amount of antibody that would have been present had the slope of the antibody curve at the time of the second injection of antigen continued until the time of the secondary peak titer. The derived rate was obtained from a line connecting the preimmunization titer with the derived peak titer.

For the initial antibody rise, three measures of the rate of accumulation were used. The first of these is k or the constant of increment during the initial antibody rise or rise to peak titer, i.e., the time from the end of the induction period to peak titer. This value, provided there were two phases of rise to the initial rise, had to be derived as an average rate of rise by obtaining the rate from a line connecting the preimmunization titer at the end of the induction period to peak titer. Closely related to this is the length of the initial rise measured in days. A third measure is k for the first or most rapid part of the antibody rise. This value was the same as the first value, when there was one rate of rise to peak titer, but was much higher if the rate of rise to peak titer consisted of two phases. It probably measures the maximum rate at which the body can form antibody under the given experimental conditions. Thus, the earlier work of the senior authors (1951b) suggests that it may be an expression of that phase of the antibody curve in which all antibody sites are active and before any such as the spleen have become inactive (W. H. and L. G. Taliaferro, 1950). The same measures were used for the secondary response, but had to be derived for rates and peak titers (see preceding paragraph).

In representative animals, the total amount of antibody formed for 30 days was calculated by the following equations (see W. H. and L. G. Taliaferro, 1951b and 1952):

For each rise in antibody where $k_1 > 0$,

$$(2) \qquad S_t = A_0 \frac{k_1 + k_2}{k_1} (e^{k_1 t} - 1)$$

where S_t represents the total amount of antibody formed at time t (serum antibody plus antibody lost by metabolic decay); A_0, the observed serum antibody at time zero; k_1, the rate of serum antibody rise; and k_2, the rate of metabolic decay which we assume to be -0.126 ($t_{1/2} = 5.5$ days).

For each antibody plateau where $k_1 = 0$, equation 2 becomes

$$(3) \qquad S_t = A_0 k_2 t$$

For each antibody decline where $k_2 < 0$ and > -0.126

$$(4) \qquad s_t = a_0 \frac{k_2 - k_3}{k_3} (1 - e^{-k_3 t})$$

where s_t represents the total antibody formed at

time t; a_0, the serum antibody at time zero; k_2, the rate of metabolic decay (-0.126) as in equation 2 and 3; and k_3, the rate of serum antibody decline. Whenever $k_3 \leq -0.126$, it is assumed that no antibody is being formed.

Total body irradiation was used exclusively. It was carried out as follows: Two rabbits were irradiated at a time in aluminum boxes at about 75 cm from the target to the center of the animal. The X-ray machine was operated at 250 kilovolts and 15 milliamperes with 0.25 mm copper and 1.0 mm aluminum filters. The half-value layer of the filtered beam was 0.9 mm copper. Radiation was delivered at the rate of 30 to 35 r per minute as measured by a Victoreen r meter after placing the thimble in the cage at about the midpoint of the rabbit's body. The beam was delivered horizontally and the cages were turned every 5 minutes to insure equal exposure. The room was air-conditioned to prevent overheating the rabbits.

The rabbits were obtained from the same source as formerly. Males and females of inbred stock were used, but were infected with both *Eimeria stiedae* and *Eimeria perforans*. We attempted to keep these infections down by the frequent sterilization of cages and general sanitary procedures, but *E. perforans* may have been responsible, in part, for the high mortality after irradiation. In addition, we found an unusually high mortality in rabbits injected with antigen from 3 to 6 hours after irradiation.

EXPERIMENTAL RESULTS

One orienting experiment and one subsequent experiment, each consisting of intact and irradiated groups of rabbits, were carried out to study the effect of X irradiation on hemolysin production during an initial response. The orienting experiment was carried out at one time, but the other experiment took several months or more to complete. Every batch of irradiated animals, however, was accompanied by two or more nonirradiated controls. A third experiment, also taking several months to complete, was carried out to study the effect of X irradiation on hemolysin production during the specific anamnestic response, i.e., after a reinjection of red cells.

Normal antibody response.—The experimental data obtained in the present work can be best interpreted in relation to the hemolysin response in nonirradiated animals which has been described in detail by the senior authors (1950, 1951b and 1952). The conclusions reached are illustrated by the controls in tables 1, 2 and 3 and may be briefly outlined as follows: After the intravenous injection of 1.25 ml of a 1% suspension of red cells/kg rabbit, the preimmunization titer of rabbits, varying from 10 to 100 units, remains unchanged or falls for several days (induction period), and the titer then rises abruptly for several days (most frequently at a single continuous rate) to the peak titer. After peak titer, antibody disappears, generally at a rapid rate for about a week or more and then at a less rapid rate for the following week or two. When two changes in the rate of the initial rise occur, the second phase is always slower than the first one. This more or less stereotyped response is also elicited by a tenth of this amount of antigen.

Within this tenfold range of amount of antigen, approximately 90% of the antibody at peak titer is produced by the spleen. At about peak titer the spleen largely ceases to form antibody, whereas nonsplenic sites continue production at a low rate. As a consequence, the relative importance of the spleen in total antibody produced (serum antibody plus antibody disappearing due to metabolic decay) decreases as time elapses. The actual units of antibody produced, however, either at peak titer or over a 30-day period, vary over a wide range from rabbit to rabbit.

Eight times this standardized amount of red cells, i.e., equivalent to 1 ml of 10% cells, yield essentially similar results, except that the initial rise is often characterized by two phases in the rate of increase, the primary antibody decline occurs at a slower rate, and splenectomy does not reduce peak titer to the

TABLE 1.—*X-ray damage (varying amounts of X rays) to the primary antibody response* after varying amounts of antigen.*

Rabbit no.	Wt. in kg	X rays	Red cells/kg injected 2 days after X rays	Hemolysin units/ml (×10⁶)		Length in days of:		Rate of accumulation (k)		Apparent half life of most rapid decline in days
				Peak titer	Total formed in 30 days	Induction period	Initial rise to peak	Initial phase	Derived when more than one phase	
195	2.7		1.25 ml 1%	0.1	0.2	10.8	3.0	0.56		9.9
196	2.5		1 ml 10%	5.3	12.0	2.8	5.2	1.1		13.1
197	3.6	400r	1 ml 10%†	32.0	104.2	3.6	14.4	1.68	0.45	6.9
198	2.4		1.25 ml 1%	0.06	0.1	7.0	21.0	0.28	0.70	
199	2.4	600r	1 ml 10%	1.6	3.2	3.2	5.2	0.84		10.3
200	3.4		1 ml 10%†	Died before peak		5.4		0.38		
201	2.8		1.25 ml 1%	0.3	0.6	6.0	9.2	0.91	0.37	4.6
202	2.7	700r	1 ml 10%	0.4	1.1	6.8	19.2	0.37	0.11	
203	3.2		1 ml 10%†	20.0	55.5	5.4	14.0	1.54	0.49	7.7
Number in group	Range in weight	Control groups: no X-rays								
52	1.7–2.5	None	1.25 ml 1%	3.0±0.35	4.2±0.43	3.0±0.11	4.6±0.2	1.23±0.05	1.03±0.04	5.7±0.3
27	1.8–2.8	None	1 ml 10%	2.9±0.31	4.7±0.59	2.7±0.09	4.8±0.1	1.12±0.07	0.87±0.04	8.0±4.0
24	1.7–2.7	None	1 ml 10%†	18.1±1.6	45.8±8.0	2.2±0.18	11.6±1.3	1.61±0.12	0.76±0.07	10.1±1.0

* Mean values ± standard errors are from fitted curves. † 17 injections during the course of 34 days.

same extent. These differences following the injection of eight times the intermediate amount of antigen were attributed to the production of additional antibody by nonsplenic sources. It is to be noted that in spite of the differences between injecting this amount and the smaller amounts, peak titer is statistically the same in all groups and varies from rabbit to rabbit over the same wide range.

Reinjection of a single dose of antigen within the 80-fold range described above, at any time from the time of peak titer to 176 days after the initial injection, results in the production of statistically similar amounts of antibody as during the primary response. The heightened reactivity usually associated with the homologous anamnestic response is limited to a reduction of the induction period by a third and a higher rate of antibody rise, which occurrs almost invariably as a single phase rise. The spleen is about as active during the secondary response after a single antigen reinjection as during the initial response.

Experiment 1.—This experiment was planned to ascertain the dose of X rays and the amount of antigen best suited to study critical timing of X-ray damage. It consisted of three sets of three rabbits which were irradiated with 400, 600 and 700 r, respectively. Two days later one rabbit in each set was injected once with 1.25 ml of 1%, once with 1 ml of 10% and 17 times during the course of 34 days with 1 ml of 10% red cells, respectively.

Table 1 gives the results along with data from three groups of nonirradiated rabbits receiving the same amounts of antigen. As may be seen by comparing each rabbit with its appropriate control group, all doses of X rays depressed hemolysin formation after injection of 1.25 ml of 1% red

cells. For example, in rabbit 195, as compared to the control group, the induction period was three times longer, peak titer was only 100 units/ml as compared to 3000 units, the rate of accumulation to peak titer was halved, and the total amount of antibody formed in 30 days was 200 units/ml instead of 4200 units. The seemingly less rapid disappearance of antibody (9.9 days as compared to 5.7 days) was probably a reflection of the continued low grade production of antibody in this irradiated animal and was frequently encountered when little antibody was formed.

Following one or repeated injections of 1 ml of 10% red cells, 400 r were ineffective in depressing antibody formation, and 600 r and 700 r, though partially effective, allowed sufficient antibody to be formed to be within the range of that formed in the control group. The course of hemolysin production in rabbits 195 and 196 in figure 1 illustrates, on the one hand, the depressing effect of X irradiation when followed by a small amount of antigen and, on the other hand, its ineffectiveness against more intense antigenic stimulation.

In view of the variable reactivity of rabbits, which has been evident in all of our work, we decided, in the following experiments, to inject the intermediate amount of red cells at various intervals before and after X irradiation with 600 or 700 r.

Experiment 2.—From 2 to 12 rabbits were injected with 1.25 ml of 1% red cells/kg at the following intervals before irradiation—4, 2 and 1 days, 18, 12, 8, 6, 3 and 2 hours, and 10 minutes—and after irradiation—3, 6, 12 and 18 hours, and 1, 2, 5, 7, 10, 14, 21 and 28 days. All times refer to the midpoint of irradiation. A group of similarly injected but not irradiated

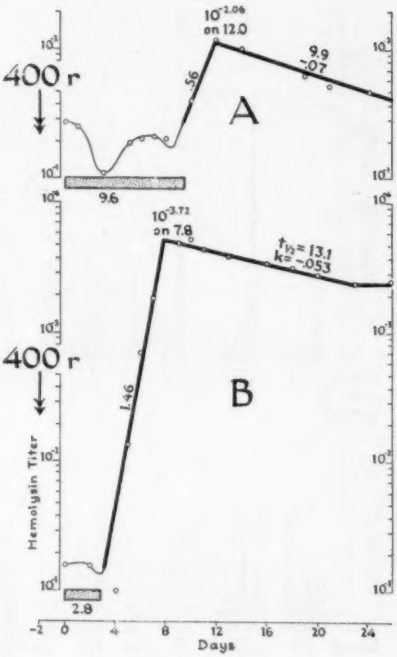


FIG. 1.—The relationship of antigen dose to X-ray damage.

A. Rabbit 195, which received 1.25 ml of 1% red cells/kg 2 days after 400 r, showed the typical effect of irradiation, i.e., a lengthening of the induction period, a slow rate of antibody rise and a low peak titer. B. Rabbit 196, which received 1 ml of 10% red cells/kg 2 days after the same amount of irradiation, showed an antibody curve indistinguishable from that of normal animals.

Zero on the abscissa axis refers to the time of antigen injection; double-headed arrow, the time of irradiation; stippled bar, the induction period with its length in days; fine line, the titer during the induction period; and heavy line, the antibody response. On each antibody curve are indicated rate constants (k) for each separate rise, peak titer and the day on which it was reached, and the apparent half life in days for the antibody decline with the value of k immediately below it.

controls accompanied each group of X-rayed animals. The irradiated rabbits totalled 134 and the controls, 58 (fig. 2). The rabbits usually weighed

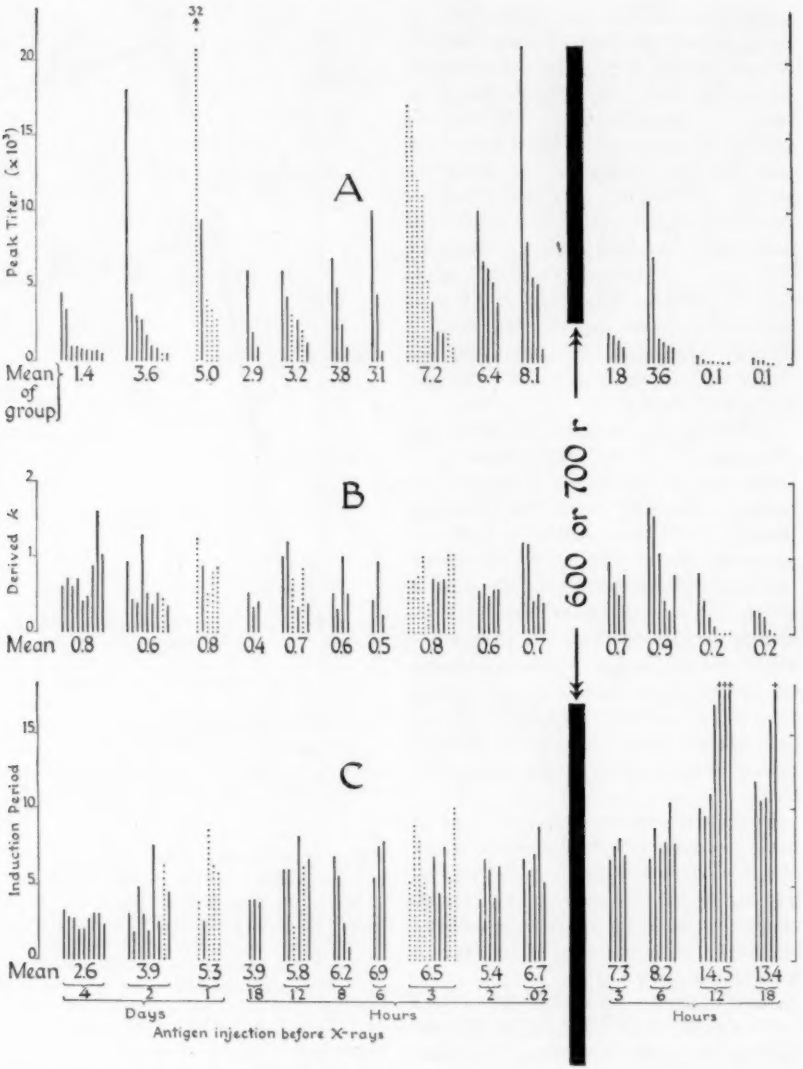
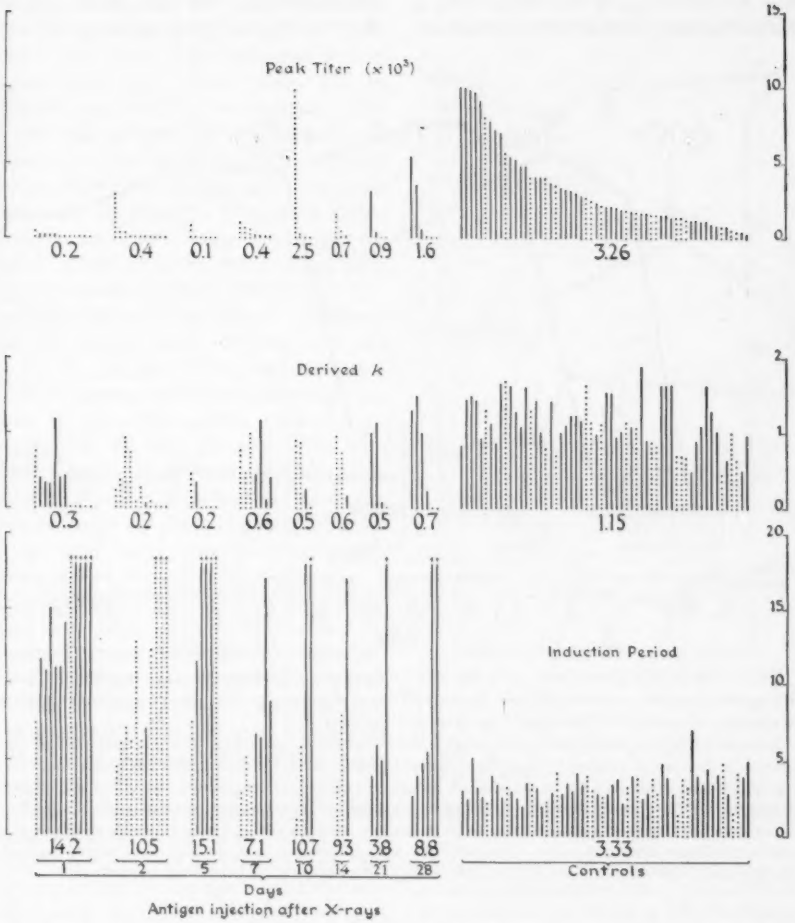


FIG. 2.—Antibody formation in 134 rabbits, injected with 1.25 ml of 1% red cells at intervals from 4 days before to 28 days after irradiation with 600 r or 700 r total body irradiation, and in 58 non-irradiated controls. Broken lines, animals receiving 700 r or their controls; solid lines, rabbits receiving 600 r or their controls.

A, peak titers which are arranged within each time interval in a decreasing order of magnitude;



B, derived values of k giving the rate of rise of antibody from the end of the induction period to peak titer; and C, length of the induction period in days. The values in rows B and C are placed directly underneath peak titers for the corresponding rabbit in row A. To obtain the means, each animal forming no antibody was given its preimmunization titer for peak titer; the value, zero, for the derived k; and an arbitrary value of 18 days for the induction period. These data are grouped differently in table 2.

between 2 and 2.5 kg. Irradiated rabbits received either 600 or 700 r. As may be seen by the distribution (cf. solid vs. dotted lines) in figure 2, there was no difference of statistical importance between the two sets of animals. Under each peak titer is indicated the rate of increase (section B) and the induction period (section C) for each individual rabbit. These same three measurements, together with values for k for the first phase of the initial rise

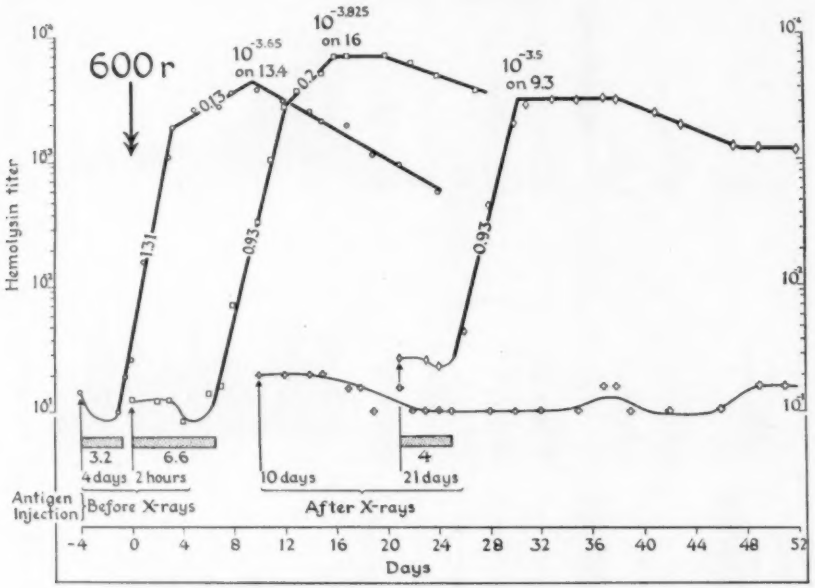


FIG. 3.—Antibody curves from 4 of the rabbits in figure 2 showing the main features of X-ray damage with respect to hemolysin and its rate and speed of formation. Zero on the abscissa axis refers to the time of irradiation. See figure 1 for other explanations.

Note in the first antibody curve, the lengthened initial rise to peak titer which is composed of two phases; in the second antibody curve, the stimulating effect as shown by a higher peak titer than normal which was attained after a lengthened induction period and lengthened rise composed of two phases; in the third antibody curve, the lack of response which is characteristic of almost a third of the rabbits injected from 12 hours after to 21 days after irradiation (see figure 4 for other variations); and, in the fourth antibody curve, the practically normal response (occasionally encountered through 21 days after irradiation and evident in half of the rabbits injected 28 days after irradiation).

They are, therefore, considered together.

Salient data from these rabbits are shown in figures 2 through 4 and in table 2, along with the group of 58 nonirradiated control rabbits. In figure 2, the rabbits receiving irradiation at each time interval are classified according to peak titer (section A) and are arranged in order of decreasing peak titer. and the length in days of the antibody rise to peak titer, are given in table 2 for various combined intervals before and after irradiation. Figure 3 gives representative antibody curves at four intervals with respect to irradiation, and figure 4 gives five variations of the hemolysin curve encountered in the group of rabbits injected with red

cells two days after irradiation, i.e., in the zone of maximum antibody inhibition. The data may be further described as follows:

Examination of the peak titers for the controls receiving no X rays indicates that peak titer extended over a wide range and varied from 230 to 10,000 units/ml. The distribution, however, was smooth, although there was some skewness due to the fact that peak titers of 38 rabbits were under the mean and only 20 above it. This skew distribution was also characteristic of the peak titers reported from 67 rabbits by the senior authors (1952).

The most striking feature of figure 2 is the sharp break between animals receiving antigen 6 hours after irradiation or before and those receiving antigen 12 hours through 21 days after irradiation. Of the 59 rabbits in this latter group, 36 formed little antibody (table 2) and 18 formed no antibody, as indicated by the fact that the hemolysin titer never rose over the preimmunization titer.* The remaining 5 rabbits (one at 2, 7, 10, 14 and 21 days) not only attained peak titers within the normal range (3660 ± 1490 units/ml) but also had normal antibody curves. Recovery was largely complete in 3 of 6 animals injected with antigen 28 days after receiving X rays (fig. 2 and table 2).

The variations in hemolysin inhibition are shown in figure 4. No hemolysin was produced in figure 4A (cf. 3rd graph in fig. 3). In the rest, little was produced within a wide range of rates ($k = 0.1$ to 0.87). In figure 4B, the rise was characterized by two phases. In figure 4E, two rates of increase were separated by a slight decline, but titers were so irregular from the 12th through the 20th day that the decline might

* Most of these rabbits were observed for 30 days but a few were observed for as long as 60 days.

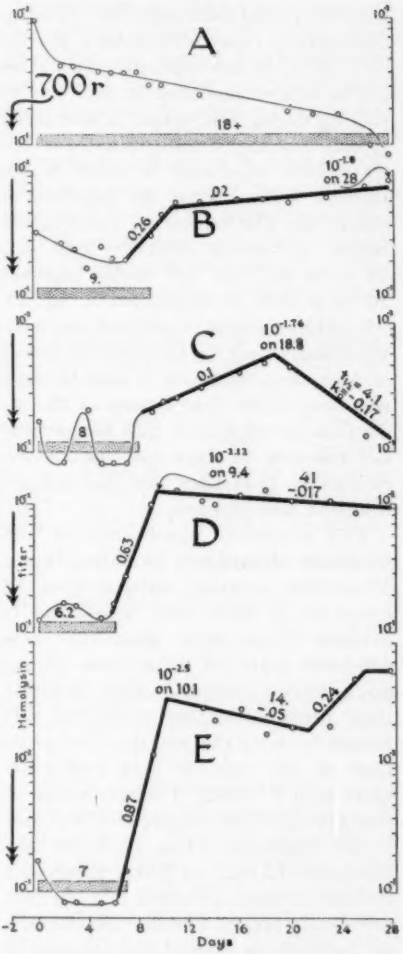


FIG. 4.—Five antibody curves taken from the group of rabbits injected with 1.25 ml of 1% red cells/kg 2 days after receiving 700 r. See figure 1 for explanations.

Curve A is representative of those animals which formed no hemolysin. In figure 2 and in table 2, such animals were arbitrarily given an induction period of 18+ days. Curves B, C, D and E variously illustrate the lengthened induction period, the low peak titer and the retarded rate of rise of antibody encountered at this time. A secondary rise (starting on about the 21st day in curve E) was occasionally found in this group. It was only rarely seen in nonirradiated rabbits.

easily have been described as a plateau. This type of curve with either a plateau or a slight decline separating two rates of rise was encountered in 5 of the 59 rabbits during this period as compared to only 1 of the 58 controls and 1 of the 69 rabbits irradiated 6 hours before through 4 days after the injection of sheep cells. The longest induction period before an actual antibody rise was 18 days (cf. the 3rd rabbit injected 10 days after X irradiation in fig. 2). All rabbits showing no antibody rise were accordingly assigned an induction period of 18+ days in figure 2, and 18 days was used in the calculations of the induction period means. The longest initial rise was 21 days and the longest interval to peak titer after the antigen injection was 38 days.

The statistical significance of the inhibition of antibody formation in the 59 rabbits receiving antigen from 12 hours to 21 days after irradiation is evident. Their mean peak titer was 470 ± 160 units/ml when those giving no antibody response were assigned their preimmunization titer. The difference between this and the mean peak titer of the controls was 2960 ± 400 units with $P < 0.001$. The occurrence of complete antibody suppression was also highly significant. Thus, when the rabbits in the 12-hour to 21-day group and the controls were arranged in a fourfold table according to whether they formed no antibody or appreciable amounts χ^2 (after Yates correction) was 19.3 with $P < 0.0001$. Associated with the inhibition of antibody formation, there was an increase in the induction period and a decrease in the rate of antibody formation. These results are strikingly shown even if the comparison is limited to the 32 rabbits which produced antibody in the group injected 12 hours to 7 days after irradiation. Dealing with this group of rabbits has two advantages. It omits the 16 rabbits which formed no antibody and for which an arbitrary figure would have to be assigned to the induction period and a zero rate of increase: it also eliminates three rabbits during the second and third week after irradiation which had largely recovered. The peak titer of this group of 32 rabbits was 360 ± 90 units with P for the difference from the normal group < 0.001. The mean induction period was 9.71 ± 0.70 days. The difference of 6.37 ± 0.55 days was highly significant, with $P < 0.001$. Similarly significant statistical differences from the normal group were found for all the measures of the rate of antibody formation and indicated a marked increase in the time of the antibody rise and a marked decrease in the value of k for the first phase and for the derived value of k (table 2).

As may be seen by an examination of the 69 rabbits which received antigen from 4 days before to 6 hours after irradiation in figure 2 and the second graph in figure 3, the immune process was significantly retarded, but the total antibody eventually produced was frequently above normal. In estimating the rate of the immune process, the following facts are pertinent: The rabbits injected 4 days and 2 days before irradiation should be excluded from a consideration of the induction period since the first group was irradiated after the induction period had passed and the second after it was two-thirds over. The remainder of the animals (51 injected 1 day before to 6 hours after) had an induction period of 3.17 ± 0.26 days longer than the controls ($P < 0.001$). As compared to the controls, the whole group of 69 animals showed a slower antibody rise as indicated by (1) a smaller value of k for the initial antibody rise (0.99 ± 0.04 with $P < 0.001$), (2) a smaller value of the derived k for the

entire antibody rise to peak titer (0.71 ± 0.04 with $P<0.001$), and (3) a longer antibody rise (8.40 ± 0.42 days with $P<0.001$). It should also be noted that calculations based upon the omission of the rabbits injected 3 and 6 hours after irradiation did not alter the significance of these differences.

The slower development and production of antibody did not prevent some rabbits from attaining peak titers higher than the top range of the controls. Although these high titers occurred sporadically in the 60 animals injected from 2 days before to 6 hours after irradiation, they more often occurred in those animals injected with antigen from 3 hours to 10 minutes before irradiation. For example, comparison of peak titer of the entire group of 69 animals with the controls showed a difference of 1350 ± 810 units/ml, which is not significant. On the other hand, comparison of the 20 rabbits injected 3 hours to 10 minutes before irradiation showed a difference of 3810 ± 1670 units/ml with $P=0.03$.

The validity of using peak titer as an indicator of antibody formed is considered in the discussion.

Experiment 3.—This experiment was designed to ascertain the effect of X irradiation on specific or homologous anamnesis, i.e., the secondary antibody response. It consisted of the following reinjections: into 12 rabbits 6 hours to 4 days before X rays; into 22 rabbits 2 days after X rays; into 23 rabbits 31 to 56 days after X rays; and into 15 nonirradiated controls (table 3).

As a basis for the interpretation of the experimental results, the senior authors (1952) have already reported the main characteristics of this response after the reinjection of the same quantity of sheep red cells per body weight. These features are well shown by comparing the response in the 15 controls receiving a second antigen injection from 7 to 97 days after the first injection in table 3 with the nonirradiated controls in table 2, which received only one antigen injection. They may be briefly noted as follows: (1) The peak titer of these two groups did not differ significantly ($P=0.6$). (2) The induction period was shortened by about one-third ($P<0.001$). (3) The antibody rise to peak titer was shortened ($P<0.001$). (4) The derived value of k

TABLE 2.—*X-ray damage to the primary hemolysin response* in relation to the time of antigen injection.*

Rabbits in group	Time of red cell injection in relation to X rays*	Peak titer ($\times 10^6$)	Length in days of:		Rate of accumulation k	
			Induction period	Antibody rise to peak	First phase	Derived when more than one phase
9	4 days before	1.44 ± 0.27	2.69 ± 0.16	6.34 ± 1.29	1.09 ± 0.19	0.83 ± 0.16
9	2 days before	3.65 ± 1.86	3.89 ± 0.67	8.99 ± 1.74	0.88 ± 0.1	0.58 ± 0.10
18	1 day-8 hr. before	5.25 ± 1.68	5.96 ± 0.54	8.42 ± 0.76	0.8 ± 0.06	0.66 ± 0.07
23	6 hr.-10 min. before	6.95 ± 1.17	6.43 ± 0.31	9.52 ± 0.81	1.06 ± 0.09	0.7 ± 0.06
10	3–6 hr. after	2.96 ± 1.0	7.80 ± 0.36	7.10 ± 0.84	1.19 ± 0.1	0.84 ± 0.14
16 32	12 hr.-7 days after	0 for 30 days 0.36 ± 0.09	9.71 ± 0.70	8.93 ± 1.11	0.60 ± 0.07	0.44 ± 0.05
2 9	10–21 days after	0 for 30 days 1.74 ± 0.96	7.51 ± 2.00	8.60 ± 2.39	0.67 ± 0.09	
3 3	28 days after	0 for 30 days† 3.09 ± 1.3	3.80 ± 0.37	3.83 ± 0.33	1.26 ± 0.12	
58	Control group: no X rays	3.43 ± 0.37	3.34 ± 0.13	4.32 ± 0.16	1.24 ± 0.05	1.15 ± 0.05

* All values are means ± standard errors and are from fitted curves. The antigen consisted of a single injection of 1.25 ml of 1% red cells/kg given from 4 days before through 28 days after total body irradiation with 600 r or 700 r.

† One of these rabbits gave a slight response, i.e., a peak of 110 units reached after 11.6 days with an induction period of 5.6 days.

was increased ($P<0.001$). These results indicate that no more antibody was formed during anamnesis but that it was formed sooner and at a faster rate.

The anamnestic response two days after irradiation, i.e., at maximal X-ray effectiveness, was studied in 22 rabbits. As shown in table 3, these were given 600 or 700 r from 28 to 85 days after receiving their first injection of antigen and two days later were reinjected with the same amount of antigen, i.e., 1.25 ml of 1% red cells/kg. The expected specific anamnestic response was inhibited just as was the initial response when antigen was given 12 hours to 7 days after irradiation (cf. table 2). The mean peak titer of the 5 animals giving a partial anamnestic response was 320 ±100 units/ml, which did not differ significantly from that in the 32 reactors of table 2 which received an initial injection of antigen from 12 hours to 7 days after irradiation. A larger proportion of the animals in the anamnestic test than in the primary response, however, gave no antibody response. Thus, when the animals in the two groups were arranged in a fourfold table according to whether or not they produced appreciable antibody and as to whether or not they received an initial or a second antigen injection, χ^2 was 7.7 with $P=0.01$.

The characterization of the rate of the immune process in the X-rayed animals has to be limited to the five which formed some antibody (table 3). Comparison of these with the 15 nonirradiated controls in table 3 gives the following differences in the secondary response associated with X irradiation: (1) The induction period was increased from 2.09 to 3.50 days, with $P=0.02$. (2) The time of the antibody rise was increased from 2.79 to 7.86 days, with $P=0.05$. (3) The derived value of k was decreased from 2.01 to 0.47, with $P<0.001$. Although the changes were similar to those found in the initial response, the percentage increase in the induction period was less than in the initial response. Such comparisons, moreover, do not take into account the greater percentage of animals showing complete inhibition of the antibody response in the anamnestic group. In any event, it is interesting that the induction period in these rabbits was lengthened by X rays to approximately the value of the initially injected nonirradiated controls.

TABLE 3.—*X-ray damage to the secondary hemolysin response* in relation to the time of antigen injection.*

Rabbits in group	Time of red cell reinjection in relation to: Initial injection	X rays*	Peak titer ($\times 10^3$)	Length in days of: Induction period	Initial rise to peak	Rate of accumulation (k): Initial phase	Derived when more than one phase
3	21–42 days after	4 days before	2.27±0.82	2.07±0.27	3.00±0.26	1.72±0.34	
3	21 days before	1 day before	0 for 30 days				
6	32–47 days after	1 day-6 hr. before	3.3 ±0.96	6.0 ±1.18	5.75±0.90	1.10±0.53	1.0 ±0.54
17, 5	30–87 days after†	2 days after	0 for 30 days; 0.32±0.1	3.5 ±0.48	7.86±2.39	0.47±0.10	
23	29–40 days after‡	31–56 days after	2.13±9.35	2.33±0.19	4.43±0.61	1.23±0.13	1.17±0.13
			Control group: no X rays				
15	7–97 days after	No X rays	3.03±0.53	2.09±0.27	2.79±0.25	2.01±0.21	

* All values are means ± standard errors and are from fitted curves. The antigen consisted of 1.25 ml 1% red cells/kg rabbit given from 4 days before through 56 days after total body irradiation with 600 r or 700 r.
† Peak hemolysin titer of initial injection was normal in all (mean = 3.5 ± 0.84).
‡ Peak hemolysin titer of initial injection was subnormal in all (mean = 0.15 ± 0.04).

A typical example of this experiment is shown in figure 5. The initial response in this rabbit after 1.25 ml of 1% red cells/kg resulted in a peak titer of 2000 units/ml, which was reached on the 7th day. The titer had fallen to 210 by the 50th day, when the animal was given 600 r total body irradiation. Two days later, 52 days after the initial antigen injection, it was reinjected with the same number of red cells/kg as at the initial immunization. There was no rise of antibody following irradiation but a continued decline. Although this decline began at about the time of irradiation, it was probably not caused directly by irradiation because such declines take place in nonirradiated animals (see W. H. and L. G. Taliaferro, 1950). The only effect that might possibly be ascribed to X irradiation was the marked irregularity of the titers for about 2 weeks after irradiation. As a further control to test its reactivity, this animal, 41 days after X irradiation, was again reinjected with the same amount of antigen. This reinjection resulted in a second peak titer of about 1000 units, which is within the range of the response of animals reinjected from 31 to 56 days after irradiation (table 3). An interesting feature of this second reinjection without irradiation was that the induction period was only 1.2 days. In other words, the secondary response without irradiation showed the typical shortened induction period.

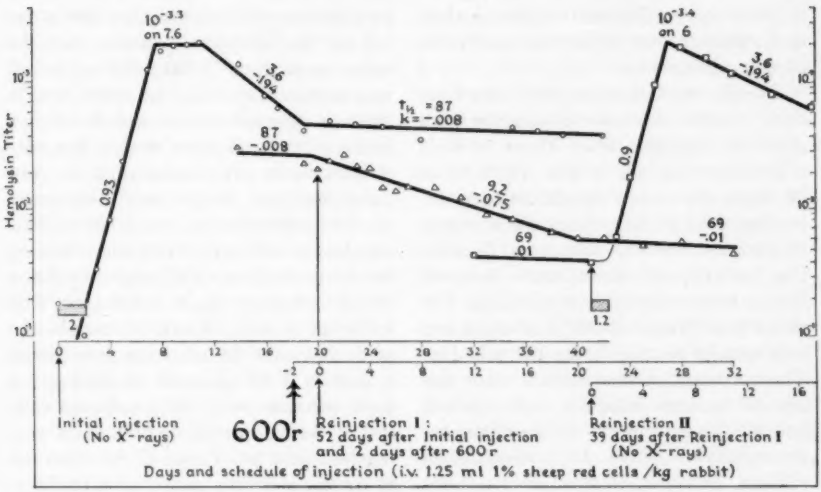

Hemolysin Titer
$10^{-3.3}$ on 7.6
$10^{-3.4}$ on 6
3.6 -.194
$t_{½}$ = 87 k = -.008
87 -.008
9.2 -.075
69 .01
69 -.01
0.93
0.9
1.2
Initial injection (No X-rays)
600r
Reinjection I : 52 days after Initial injection and 2 days after 600 r
Reinjection II 39 days after Reinjection I (No X-rays)
Days and schedule of injection (i.v. 1.25 ml 1% sheep red cells /kg rabbit)

FIG. 5.—X-ray inhibition of the first specific anamnestic response, as compared to (1) the normal initial response before irradiation and (2) the normal second specific anamnestic response 41 days after irradiation. See figure 1 for explanations.

Three rabbits were reinjected 4 days before irradiation and gave an essentially normal response (table 3). Six of the 9 animals reinjected 1 day to 6 hours before irradiation attained normal peak titers but with the characteristic slowing of the immune process noted for the primary response. The remaining three did not form appreciable quantities of antibody ascribable to a secondary response. A χ^2 test in a fourfold

table indicated that the number of animals giving no response was probably significant ($P=0.05$) in comparison to the 21 rabbits in table 2 which received antigen from 1 day to 6 hours before irradiation. Although the total number of rabbits was small, the nonreactors in this group as well as the ones in the group reinjected 2 days after irradiation indicate that the anamnestic response is more susceptible to irradiation than is the primary one under the conditions of our experiments.

Twenty-three animals were tested for their specific anamnestic response with a second injection from 31 to 56 days after receiving 600 r and from 29 to 40 days after their initial antigen injection. The primary response in many of these rabbits was subnormal because the majority of them were X-rayed 2 days before the antigen injection. The mean peak titer was 247 ± 60 units and was usually reached after the 9th day. The recovery of the rabbits after the second antigen injection was marked (table 3). As compared to the anamnestic response in the 15 nonirradiated rabbits, however, it was probably not complete, as indicated by (1) a somewhat lower mean peak titer (2130 ± 350 units), (2) a slower accumulation of antibody in the serum (1.17 ± 0.13) and (3) a longer antibody rise (4.43 ± 0.61 days). These observations are more or less in accord with the recovery of rabbits when injected initially 28 days after X irradiation (table 2).

DISCUSSION

Our measures of the effect of X irradiation on antibody response are of two types: (1) the amount of antibody formed as indicated by the peak serum titer and (2) the speed of the immune process as indicated by the length of the induction period and the length and rate (actual and derived values of k) of the antibody rise to peak titer. Logically, it would be expected that the amount of antibody formed would be directly correlated with the speed of the immune process. Actually, a general slowing of the immune process was not necessarily associated with a lowered antibody formation. Thus, in animals injected with antigen 3 hours to 10 minutes before irradiation, all of the rate measures indicated a marked slowing of the immune process, but the mean peak titer (7240 ± 940 units/ml) was remarkably high. In other words, animals injected before and through 6 hours after irradiation with a low rate of formation often continued to produce antibody longer and eventually reached above normal peak titers. This conclusion is illustrated by the following mean values from two groups of rabbits which attained peak titers of 9000 units/ml or more. From 5 controls, the peak titer of 9700 units was attained at a rate of 1.24 (derived k) during 5.1 days, whereas from 11 irradiated rabbits a peak titer of 15,200 units was reached at a lower rate of 0.9 (derived k) during a longer period of 8.15 days.

The present work indicates that there are two effects of large doses of X rays on antibody formation: (1) the complete or almost complete suppression of antibody formation which is associated with a very short radiosensitive stage at the beginning of the immune process and (2) a general retardation of the antibody process which is not only not necessarily incompatible with the formation of normal amounts of antibody but is associated with above normal peak titers in some animals. The radiosensitivity of the processes leading to the second effect is not limited to a short initial phase of the immune process and may extend throughout the entire active immune process.

The localization of that type of X-ray

injury which markedly inhibits antibody formation to an early stage of the immune process and, conversely, the separation of the immune process into a radiosensitive early phase and a radioresistant production stage is shown by the following considerations regarding the power to produce antibody. The data in table 2 indicate (1) that there is no dimunition and at times there is an actual stimulation in the amount of antibody produced when antigen is injected before and up to 6 hours after X irradiation and (2) most animals injected before irradiation produced large amounts of antibody during the time when X-ray damage was at its height, i.e., within 9 days after irradiation (cf. Bloom, 1948, and collaborators). It is, therefore, evident that the power to produce antibody is not affected by X irradiation and that antibody formation is inhibited during a short initial radiosensitive period of the immune process. This sensitive period is probably not more than 6 hours long in view of the following analysis of our results. There was a latent period of 6 hours between irradiation and the manifestation of injury since animals injected with antigen 6 hours after irradiation formed normal amounts of antibody. Antibody inhibition was in full effect in animals injected 12 hours after irradiation.

The slowing of antibody formation (an increased length of the induction period and rise to peak titer, and a decreased value for k without a marked reduction in peak titer) probably took place in all groups injected with antigen from 1 day before to 6 hours after irradiation. It also occurred in those injected 2 days before irradiation, except for a lengthening of the induction period which was almost over by the time of irradiation. Similarly, the antibody rise was somewhat retarded in the group given antigen 4 days before irradiation, as indicated by the small derived value for k ($P = 0.05$ as compared to the control group). This low rate with no significant lengthening of the antibody rise probably accounted for the low mean peak titer in this group. It seems not improbable that high doses of X rays elicit this retarding effect when administered at any time during the immune process but obviously cannot affect a stage already passed and produce progressively smaller changes as the rate of antibody formation declines. The last fact may explain why the senior authors (1950b) found no change in the character of the antibody decline following 2 doses of 400 r administered at 2 and 4 weeks after a single injection of red cells. It may also account for results by other investigators who similarly reported a failure to alter the immune response with X irradiation after the immune response had been in progress for varying lengths of time (see review in W. H. and L. G. Taliaferro, 1950; and especially Craddock et al, 1948 and 1949; Clemmesen, 1948; and Burrows, 1948).

It is impossible to differentiate or determine the relation of the slowing processes involved in the following two groups of rabbits: (1) those receiving antigen from 4 days before to 6 hours after irradiation and (2) those receiving antigen from 12 hours to 7 days after irradiation. Antibody accumulation in the second group showed all gradations from no retardation to complete inhibition. Thus, the group contained 16 animals in which the rate was reduced to 0 and 32 animals in which the derived value of k ranged from 0.1 to 1.64 with a continuous distribution of the intervening values. There is nothing in our data, however, which contradicts the idea that the first retardation is a general change, possibly in all phases

of serum protein synthesis, which is independent of the decrease in antibody production provided effective antigenic stimulation is received. The actual extinction or marked inhibition of antibody formation may, on the other hand, be due to a blocking of effective antigenic stimulation—possibly by preventing the localization of antigen in antibody-forming sites and/or the early metabolism of antigen. In support of this conclusion, we have found that rabbits formed almost normal amounts of antibody, after a delay, when given 10 injections of 8 times the amount of antigen used in these experiments over a period extending from 2 to 17 days after irradiation, i.e., during the period of maximum inhibition (preliminary report, 1952b). This experiment and others with a single large injection of 5 ml of 50% cells/kg will be published later. We interpret these results as indicating that the injection of large amounts of antigen supplies adequate antigenic stimulation.

TABLE 4.—*Comparison of mean values* for peak hemolysin titer and total antibody/30 days in three groups of irradiated rabbits and one group of non-irradiated controls.*

Rabbits in group	Time of red cell injection in relation to X rays	Peak titer ($\times 10^9$)	Total antibody/30 days ($\times 10^9$)	Peak titer as per cent of total antibody
4	4 days before	2.30	3.80	61
40	2 days before—6 hours after	5.68	11.34	50
22	12 hours—21 days after†	0.22	0.47	47
54	Control group: no X rays	3.42	5.39	63

* All titers (hemolytic units/ml serum) are from fitted curves. These rabbits from experiment 2 were given 1.25 ml 1% red cells from 4 days before through 21 days after total body irradiation with 600 r or 700 r.

† Limited to rabbits producing appreciable amounts of hemolysin but omitting 5 which had a short induction period and a peak titer within the normal range.

The slowing of the immune process in the animals given antigen from 4 days before to 6 hours after irradiation was associated with a higher than normal proportion of two-phase rises of antibody to peak titer. Thus, 36 of these 69 X-rayed animals had double phase rises as contrasted to 8 of the 58 controls. A χ^2 test of these results arranged in a fourfold table gave a probability of <0.0001. In view of the earlier work of the senior authors (1950), this result may indicate that the spleen stops forming antibody earlier relative to the peak titer in the irradiated animals. The animals which formed appreciable amounts of antibody among those receiving antigen from 12 hours to 21 days after irradiation did not have as high a proportion of double phase rises. This result may be associated with the small amount of antibody formed.

Antibody formation in the foregoing account has been considered to parallel peak titers. When the rates of accumulation are approximately the same, this procedure is warranted, as has been shown by the senior authors (1952). In using equations 2 and 4, however, it is obvious that the lower the rate of rise (k_1) or the lower the rate of fall, i.e., as $k_3 \rightarrow 0$, of antibody, the larger is the relative importance of the component of total antibody which is lost by metabolic decay. As a consequence, comparisons of peak titers would somewhat increase the difference between the controls and the markedly inhibited animals injected from 12 hours to 21 days after irradiation and would decrease the difference between the controls and those injected from 4 days before to 6 hours after irradiation. To illustrate this conclusion, table 4 contains mean values for peak titer and for total antibody for 30 days for 3 groups of irradiated rabbits and the control group. (Only data from rabbits living 30 days or longer were used here.) As may be seen in this table, serum antibody at peak titer accounted for practically the same percentage (63 and 61%, respectively) of

total antibody/30 days in the controls and in the animals injected with antigen 4 days before irradiation. In this last group as mentioned previously, irradiation did not lengthen the induction period and only moderately decreased the rate of accumulation. In contrast, peak titer accounted for only 47% of the total antibody in the group showing maximal X-ray inhibition, e.g., in those receiving antigen from 12 hours through 21 days after irradiation. This difference does not, however, affect the significance of the difference previously noted, especially since it does not take into account one-third of the animals which formed no hemolysin at all. Since peak titer accounted for only 50% of the total antibody in the group receiving antigen from 2 days to 6 hours after irradiation, the stimulation in antibody formation already noted for this group would be even more important from the standpoint of total antibody formed.

The decrease in the percentage of total antibody accounted for by the serum antibody present at peak titer in the two irradiated groups receiving antigen from 2 days before to 21 days after irradiation would have been greater but for the increased length of the induction period. For example, peak titer only accounted for 41% instead of 47% of the total antibody in the group given red cells from 12 hours through 21 days after irradiation, when the lengthened induction period in each rabbit was compensated for by adding the antibody formed during an equivalent number of days at the end of the 30-day period. On the other hand, the peak titer accounted for a higher percentage of the total antibody formed in both irradiated and nonirradiated rabbits with peak titers in excess of 9000 units/ml. The data are as follows: In 7 rabbits in the group injected with red cells from 2 days before to 6 hours after irradiation, their mean peak titer of 17,600 units accounted for 59% of their total antibody as compared to 43% in the remaining 33 rabbits with a mean peak titer of 3150 units. In 5 control rabbits, their mean peak titer of 9700 units accounted for 70% of their total antibody as compared to 61% in the remaining 49 rabbits with a mean peak titer of 2780 units.

As regards the relation between the time of irradiation and the effect on antibody production, our work largely corroborates the findings of Dixon et al (1951), who studied complement fixation (determined in 50% units) in rabbits following immunization with I^{131}-labelled bovine γ globulin and especially Dixon et al (1952), who studied the rate of antigen disappearance and the maximum precipitin response in rabbits similarly immunized. In the first paper, the authors divided the immune response into a radiosensitive and radioresistant phase and made the following suggestions and conclusions: The radiosensitive stage lasts only a few hours and represents the stage in which a lasting orientation of antibody-producing sites to the specific antigen takes place. The radioresistant stage is the stage during which antibody is produced. X-ray injury produces the loss of some substance during the radiosensitive stage which is necessary for antibody formation. A secondary response, following a reinjection of the specific antigen 40 hours after irradiation, is radioresistant. The authors interpreted this finding to indicate that there is a lasting orientation of the antibody-forming sites to the specific antigen such that the processes taking place during the radiosensitive stage are unnecessary after reinjection, i.e., during the anamnestic response. These conclusions were amplified in the second paper. The radiosensi-

tive stage is limited to 12 hours or less during which an adaptation of globulin synthesis to the new antigen may occur.

The most striking difference between our findings and those of Dixon et al is that we found that the homologous anamnestic response following a second injection is fully as, if not more, susceptible to X irradiation than the initial response (see exp. 3). Assuming that this difference lies in some factor of immunization such as the type or amount of antigen, an earlier stage of the immunization response is probably damaged under the conditions of our experiments.

The retardation of the immune response, with the production of normal or above normal amounts of antibody, in animals injected with antigen before or up to 6 hours after irradiation could hardly have been found by the methods employed by most of the earlier workers. Our results are, however, in essential agreement with those of Kohn (1950), who studied antisheep hemolysins in the rat and obtained antibody curves using 50% hemolytic units photometrically determined. He reported that X-ray damage occurs after as well as before injecting antigen. He described the maximum effect, which occurred only when irradiation was given before the injection of antigen, as a reduction and delay in peak titer and a plateau effect induced by a delay in the decline from peak. When an animal was injected with antigen 1 or 2 days before irradiation with 350 r or 2 to 4 days before 350 or 600 r, he found that peak titer was not greatly reduced but was markedly delayed. In the latter groups, he found that the titer of the irradiated animals eventually surpassed that of the controls under certain conditions. This finding is apparently due more to a displacement of the delayed antibody curve in the irradiated animals than to higher peak titers. With this exception his results are similar to ours. In explaining the greater effect of X irradiation given before rather than after the antigen, he suggests as a possibility that "initial events centering upon the fixation of antigen by the cells might be more sensitive to radiation than those that follow." This is somewhat similar to our suggestion that it is due to preventing localization of antigen in antibody-forming sites and/or deranging the early metabolism of the antigen.

The fact that animals irradiated from 10 minutes to 1 day after the injection of antigen were stimulated to form more antibody ($P = 0.03$) is particularly interesting. In general, previous workers who reported an enhancement of various acquired immune phenomena used much smaller doses of X rays than we used or than other workers used who reported a decrease in immunity. We have no explanation for this stimulation at present or its relationship to the time of antigen injection and irradiation. It is, however, largely localized to animals injected just before the critical stage (6 to 12 hours after irradiation) when X-ray damage inhibits the radiosensitive early stage of the immune process. It suggests the type of metabolic stimulation often observed with low concentrations of cellular poisons or during the early action with high concentrations. It may also be related to the transitory increased excretion of urinary and fecal antibody reported by Burrows et al (1950) in guinea pigs when immunization was begun a day after irradiation.

As noted earlier, large amounts of antibody were produced during the first 9 days in many animals injected before irradiation. At this time, practically all of the lymphocytes are destroyed, according to the findings of Bloom (1948) and his collaborators. These re-

sults suggest that the lymphocytes are not involved in antibody formation, although the present evidence does not exclude one or both of the following possibilities: (1) The lymphocytes play an unsuspectedly early role in the preparatory process which precedes actual antibody formation. (2) Only the comparatively small number of large lymphocytes ("young plasma cells") and small lymphocytes which survive X irradiation (cf. Bloom, 1948) are active in antibody production.

CONCLUSIONS

1. Rabbits were initially injected intravenously with 1.25 ml of 1% sheep red cells/kg at intervals from 4 days before to 28 days after a single exposure to 600 or 700 r total body X irradiation. Rabbits were similarly reinjected 4 days before to 56 days after 600 or 700 r total body X irradiation. These two phases of the work involved 134 and 57 rabbits, respectively, with adequate controls. Hemolysin was determined photometrically in 50% units at frequent intervals. X-ray damage was characterized by determining antibody formation (peak titer) and the rate of the immune process, i.e., length of the induction period and of the antibody rise to peak titer and rate constants for the first order equation describing the antibody rise. Two manifestations of X-ray injury were correlated with the time of irradiation in relation to the injection of antigen: (1) When antigen was injected from 12 hours to 21 days after irradiation, the immune process was markedly inhibited. (2) When antigen was injected from 4 days before to 6 hours after irradiation, the immune process was slowed but antibody formation was not lowered.

2. X irradiation maximally inhibited antibody formation in 48 rabbits receiving antigen 12 hours to 7 days after irradiation. No hemolysin was formed in 16 of them and peak titers of less than a ninth of the nonirradiated controls were attained in the remainder with a marked slowing of the rate of formation. This type of X-ray damage was localized to a critical stage of probably not more than 6 hours at the beginning of the immune process, as shown by the fact that rabbits injected 6 hours after irradiation formed normal or above normal amounts of antibody, whereas those injected 12 hours after irradiation formed little or no hemolysin. It is suggested that this critical phase may be related to the localization of antigen in the antibody-forming sites and/or the early metabolism of the antigen. The ability to produce antibody was practically recovered in 3 of 6 rabbits receiving antigen 28 days after irradiation.

3. X irradiation also moderately slowed the immune process in 69 rabbits receiving antigen from 4 days before to 6 hours after irradiation but did not prevent the rabbits from forming normal or above normal amounts of antibody. Peak titers increased most markedly in the rabbits receiving antigen 3 hours before to 10 minutes before irradiation. This type of X-ray damage may take place when large doses of X rays are given at any stage during antibody production. Many of this group of rabbits formed these large amounts of hemolysin during the period of marked histological damage.

4. The specific anamnestic response was as susceptible to X-ray damage as the initial response. Of 22 rabbits receiving a second antigen injection 2 days after irradiation, no hemolysin was formed in 17 of them and only small amounts were formed at a decreased rate in 5 of them. The ability to elicit the anamnestic response was largely recovered in 23 rabbits receiving antigen

31 to 56 days after irradiation. X irradiation also retarded antibody production in 9 rabbits reinjected 1 day to 6 hours before irradiation but peak titers within the normal range were attained in two-thirds of them. Three rabbits reinjected 4 days before irradiation gave a response within the normal anamnestic range.

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NUCLEIC ACIDS IN PLASMODIA AND THE PHOSPHORUS PARTITION OF CELLS INFECTED WITH *PLASMODIUM GALLINACEUM*

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The difficulty encountered in demonstrating nuclei and nuclear structure in plasmodia is fairly well known. The results obtained following the usual methods of fixation and staining have been summarized by other investigators.[1,2] However, cytochemical studies of the presence and distribution of nucleic acids in plasmodia have been limited to a few investigations utilizing the Feulgen nucleal technique. Although one investigator[3] could demonstrate no desoxyribose nucleic acid in *Plasmodium falciparum* or *Plasmodium vivax*, other investigators, using the same technique or slight variants of the technique, have demonstrated its presence in some stages of *Plasmodium relictum*,[4,5] *Plasmodium gallinaceum*,[6,7] *Plasmodium elongatum*,[8] *Plasmodium knowlesi*,[2] and *P. vivax*.[2] The results obtained in these studies vary considerably but do agree, in part, that the nuclei of certain stages of the parasite, i.e., late trophozoites, early schizonts, and gametocytes, do not stain or stain very slightly. The nuclei of other stages, i.e., older schizonts, segmenters, and large exoerythrocytic stages, apparently have greater concentrations of desoxyribose nucleic acid and are more readily seen.

No investigations of the presence or distribution of ribose nucleic acid in plasmodia are known to this author although its presence is strongly suggested by the basophilia of the organisms with Giemsa's stain and the hematoxylin-eosin-azure II technique. It is further suggested by a single report that the parasite cytoplasm is pyronin-positive.[9] This last observation was made prior to the recognition of the affinity of pyronin for ribose nucleic acid.

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Quantitative studies of nucleic acids in plasmodia have likewise received little attention. Data are available only for total nucleic acid phosphorus in *P. knowlesi* infections and for cultures of erythrocytes infected with this species.[10] In this latter study, other phosphorus (P) fractions are also considered in detail.

The present work attempts to show by staining techniques the presence and distribution of nucleic acids within the plasmodia, and to measure nucleic acids and other P fractions quantitatively by P partition of infected cells.

MATERIALS AND METHODS

Erythrocytic and exoerythrocytic stages of *P. gallinaceum* were most extensively studied. Observations on these forms were supplemented by those made on erythrocytic stages of *P. lophurae* and *P. vivax* and on preparations of sporozoites of the latter species.

Blood, brain, and sporozoite smears were immediately wet-fixed in absolute methyl alcohol, 95% ethyl alcohol, Zenker-formol solution, or formalin-alcohol-acetic acid mixture "FAA" (5 pts formalin : 90 pts 80% ethyl : 5 pts glacial acetic acid). Various time periods were used for each fixative, and in all instances 30 minutes was found to be satisfactory. Air-dried, methyl-fixed smears were also prepared for comparison and were stained with Giemsa's stain. Portions of brain containing exoerythrocytic forms were also fixed in Zenker-formol, FAA, and cold acetone. These were embedded in paraffin and sectioned at 5μ for comparison with the smear material. In addition to these methods of fixation, a wide variety of aqueous and alcoholic fixatives containing metallic salts were used. These salts included lanthanum, as recommended by Hammarsten.[11]

Treatment of slides prior to staining included the removal of malarial pigment and lipids by the use of 1:1 absolute methyl alcohol/chloroform at 60 C for 4 to 12 hours. Pigment was also removed by treatment with alkaline alcohol as described by Deane.[2] Slides were treated with crystalline ribonuclease and desoxyribonuclease (Worthington Biochemical Laboratory), using the methods described by Brachet and Shaver[12] and Stein and Gerarde[13] as well as using the enzymes in phosphate and veronal buffer systems.

The staining techniques used included the following: toluidine blue O (0.5% aqueous) for two hours, Unna's methyl green-pyronin for 4 to 6 minutes, Feulgen nucleal technique with 15-minute hydrolysis at 50 C in 1*N* hydrochloric acid and two hours in leucofuchsin, Hotchkiss alcoholic periodic acid leucofuchsin,[14] Gomori alkaline phosphatase method,[15] and Giemsa's stain.

For reasons which will be mentioned later, two methods of dehydration were used following both the toluidine blue stain and the methyl green-pyronin technique. In one, the slides were rapidly passed directly from the stain through acetone and xylene series while in the other the slides were rapidly passed through an ethyl alcohol series starting with 95% ethyl alcohol.

Fixed, unstained *P. gallinaceum* material was examined with a quartz microscope using a fluorescent screen and ultraviolet light at 2650 Å and 2804 Å. Living erythrocytic forms were also examined with a phase microscope and with a fluorescent microscope. In the latter case the parasites were vitally stained prior to examination by a ½ hour exposure to a solution of acridine O injected intravenously into the host (1.5 cc of a 1:1000 solution of acridine O in 0.85% saline per 100 g of body weight).

In all experiments where variation in prestaining treatment, staining time, and dehydration are compared, control untreated slides were included.

The phosphorus (P) partition of cells infected

10. Ball, E. G., McKee, R. W., Anfinsen, C. B., Cruz, W. O. and Geiman, Q. M. 1948, Studies on malarial parasites. IX. Chemical and metabolic changes during growth and multiplication in vivo and in vitro. J. Biol. Chem. **175**: 547–571.
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13. Stein, R. J. and Gerarde, H. W. 1951, Cytologic demonstration of nucleic acids in tissue cultures. Science, **111**: 256–257.
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15. Gomori, G. 1941, The distribution of phosphatase in normal organs and tissues. J. Cell. & Comp. Physiol. 17: 7183.

with *P. gallinaceum* was carried out by the methods devised by Schneider [16] and by Schmidt and Tannhauser.[17] The technique used for the partition of the cold acid soluble fraction into inorganic and organic fractions is that described by Friedkin.[18] The fractions prepared were total P, cold acid soluble P, inorganic and organic fractions of cold acid soluble P, lipid P, total nucleic acid P, protein P, desoxyribose P, and combined ribosenucleic acid and protein P. Ribosenucleic acid was determined by difference between fractions, as described by Kozloff.[19] The chicks used in the study of P fractions of the brain were all between 175 and 250 g in weight. Brain infected with exoerythrocytic forms was removed, kept at 0 C, weighed, and homogenized in a Potter-Elvehjem grinder at 0 C in 5% trichloroacetic acid. Aliquots were then taken for total P and for fractionation. All samples were tested in quadruplicate and figures given are average figures for each determination. An attempt was made to use birds which were of approximately the same weight and age, and which were judged to be at a terminal stage in the exoerythrocytic infection. In the analyses of normal and infected blood, the blood was drawn into 4% cold citrated saline and washed twice in cold saline. The buffy coat was removed and the cell suspension was adjusted to a suitable volume with cold saline. From this suspension suitable aliquots were removed for the various fractionations. After final volume adjustment, samples were taken for erythrocyte counts and 16 hemocytometer counts were made on each sample. In every instance the infected birds were in the initial rise of the parasitemia and no determinations were made of any infections containing more than 2% reticulocytes. The Gomori[20] modification of the Fiske-Subbarow method was used for P analysis.

RESULTS

Staining techniques

Desoxyribose nucleic acid.—The results of Feulgen staining of the parasite were judged by comparing the appearance of the parasite nuclei with those of normal host nuclei. Wet-fixed preparations were in every instance superior to air-dried smears. In examining wet-fixed films, care was taken to avoid the peripheral areas where the distortion of the parasites and the host cells was comparable to that found in air-dried preparations. In air-dried smears, host nuclei were never well stained and parasite nuclei, when visible, were always so lightly colored as to be barely perceptible. Methyl-, ethyl-, and FAA-fixed preparations were all satisfactory though Zenker-formol-fixed material was more diffusely stained. Following the methods of fixation herein reported, treatment of erythrocytic smears with alkaline alcohol as described by Deane[2] not only removed pigment but also resulted in a more diffuse stain of parasite nuclei. In addition, the nuclear granules of the host cell were slightly enlarged and there was a staining reduction in some stages of the parasite. Removal of pigment with warm methyl alcohol-chloroform mixture did not impair the staining or otherwise alter the morphology of the parasite as seen by this or other staining techniques. The absence of pigment made it much easier to see the stained nuclei.

Treatment with protease-free ribonuclease following all fixation methods had no effect on the Feulgen nuclear stain. However, exposure to desoxyribonuclease removed all Feulgen-positive

16. Schneider, W. C. 1945, Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. **161**: 293–304.
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20. Gomori, G. 1942, A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. & Clin. Med. **27**: 995.

material from both host and parasite nuclei in ethyl- and methyl-fixed slides. In FAA-fixed material the enzymes were not uniformly active even when slides were thoroughly washed in running water for a period of two to four hours. Zenker-formol-fixed slides were unsatisfactory for enzyme treatment as the enzymes were apparently inactivated.

The nuclear picture of well-fixed erythrocytic and exoerythrocytic *P. gallinaceum* following the Feulgen technique is at variance with that seen in the usual air-dried Giemsa preparations. This is particularly true of late trophozoites and early schizonts. In air-dried smears these young forms with large nuclei are badly distorted and extremely irregular in form and outline. The nuclei of these same stages in wet-fixed smears are almost uniformly spheroid, measuring approximately 1.8 to 2 μ in diameter. In later stages, the nuclei are smaller and this diameter decreases progressively to 0.8 μ or less in older schizonts and segmenters. The morphology is relatively uniform except for those stages interpreted as being division forms. Immediately prior to the last division the nuclear material is elongated and in some instances seems to be separated into two connected, roughly bar-shaped masses. In general, as the parasite progresses through the schizogonic series there is an increase in the intensity of the Feulgen reaction. In accordance with this, the nuclei of merozoites and of some minute trophozoites give the appearance of small, almost solid spheres, so deeply stained as to appear refractile. In well-stained nuclei there is a lighter area at the periphery of the nucleus or in the central portion of the nuclear sphere. It is to be noted that in no instance was there any evidence of the concentration of Feulgen-positive material in discrete particles or granules. As reported by other authors, the gametocyte nuclei could never be clearly seen and identified with confidence following the Feulgen reaction. These features of the nuclei of exoerythrocytic parasites are illustrated by figures 1 through 4 of plate I.

After treatment with ribonuclease, which completely eliminates the basophilic cytoplasmic staining of the parasite, and staining with toluidine blue O, methyl green-pyronin, or Giemsa, the nuclear morphology is very comparable to that seen following the Feulgen stain. With toluidine blue O, the nuclei of both the host and the parasite stain orthochromatically. The young stages have large, light-staining nuclei, and the intensity of the staining reaction increases progressively with the decrease in nuclear diameter (see fig. 5–8, plate I or colored fig. 1–8, plate II). In a similar manner, after ribonuclease treatment followed by methyl green-pyronin stain, the nuclei take a clear green stain which varies in intensity with the stage of the parasite. In the case of each of these two staining techniques, ordinary alcohol dehydration completely removes the nuclear stain of the parasite even though the nuclei of the host cells are still relatively

PLATE I

Exoerythrocytic stages of *P. gallinaceum* in capillary endothelium of cerebellum showing desoxyribosenucleic acid and alkaline phosphatase distribution. Figures 1–8 wet-fixed with FAA; figures 9–15 fixed in cold acetone.

FIG. 1–4.—Feulgen nucleal reaction; all stages from a single slide.

FIG. 5–8.—Ribonuclease treatment followed by toluidine blue, acetone dehydration; all stages from a single slide.

FIG. 9–15.—Alkaline phosphatase reaction; all stages from a single slide.

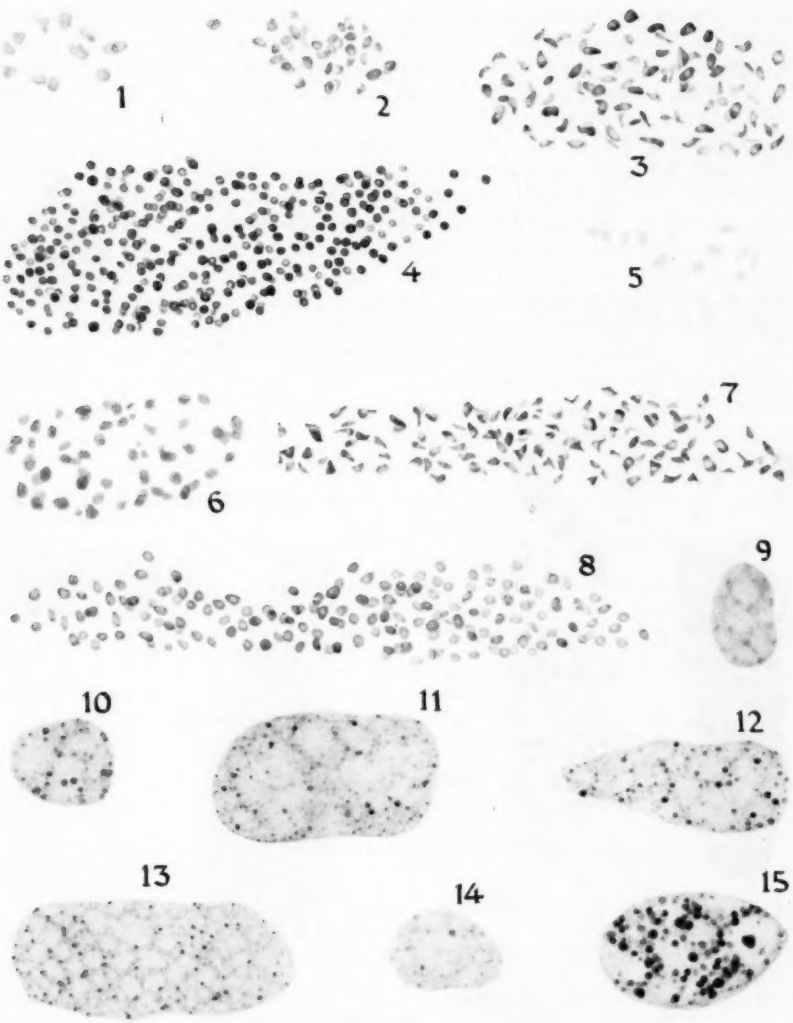

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FIG. 1, 2, 5, 6.—Entire young schizonts.

FIG. 3, 7.—Portion of schizont with dividing nuclei.

FIG. 4, 8.—Portion of more mature schizont.

FIG. 9–14.—Schizonts with alkaline phosphatase distribution closely parallel to that of ribosenucleic acid.

FIG. 15.—Alkaline phosphatase reaction as seen in a few schizonts.

All drawings were made by E. Bohlman Patterson with the aid of a camera lucida at ×3000.

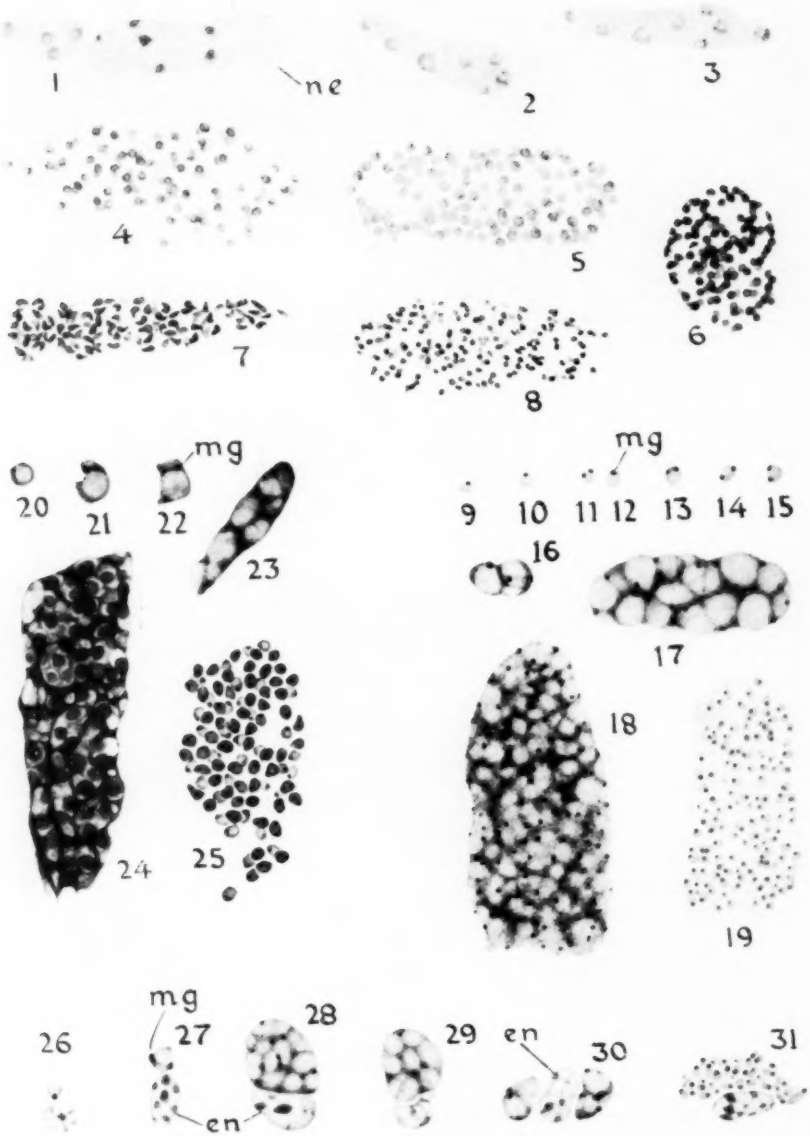
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PLATE II

intensely stained. Rapid alcoholic dehydration allows stain to remain only in the older stages. In contrast, rapid dehydration through an acetone series permits the nuclei of the younger stages to be seen although they are but faintly stained as compared to host nuclei.

Although parasite nuclei are distorted in air-dried smears, some slides prepared in this way were treated with ribonuclease and stained with the usual Giemsa technique. A series of nuclei stained by this method are illustrated in figures 1 through 25 of plate III. The sequence of varying stain intensity in these nuclei is comparable to the series obtained by the other methods. The nuclei of gametocytes and of young stages, although pale, were more readily seen than those of wet-fixed slides. Because of the compression and flattening of the nuclei by air-drying, one feature observed following the stains previously discussed is even more readily demonstrated. That is, in most nuclei a hole or a notch appears after ribonuclease treatment. This nonstaining structure varies in size from stage to stage, and corresponds in size and position to the ribosenucleic acid containing granules that will be described later. Gametocytes present a remark-

PLATE II

Nucleic acid distribution in exoerythrocytic and erythrocytic stages of *P. gallinaceum* as shown by toluidine blue.

FIG. 1–8.—Series of exoerythrocytic schizonts, young to mature, from a single slide wet-fixed in FAA, treated with ribonuclease, 0.5% toluidine blue, acetone dehydration.

FIG. 9–19.—Series of exoerythrocytic schizonts, young to mature, from a single slide, wet-fixed in FAA, 0.5% toluidine blue, alcoholic dehydration.

FIG. 20–25.—Series of exoerythrocytic schizonts, from a single slide, wet-fixed in FAA, 0.5% toluidine blue, acetone dehydration.

FIG. 26–31.—Erythrocytic series from a single slide, wet-fixed in ethyl alcohol, pigment removed with methyl/chloroform, 0.5% toluidine blue, alcoholic dehydration.

FIG. 1–3.—Young schizonts showing diffuse pale nuclei.

FIG. 4–6.—Older schizonts showing increasing concentration of Dna and decrease in nuclear size.

FIG. 7.—Portion of a schizont; dividing nuclei.

FIG. 8.—Portion of a mature schizont; many nuclei showing unstained area.

FIG. 9–15.—Young exoerythrocytic stages with metachromatic granules, cytoplasmic metachromasia, essentially unstained nuclei.

FIG. 16.—Stage with four nuclei, three metachromatic granules visible.

FIG. 18.—Portion of older schizont.

FIG. 19.—Portion of mature schizont.

FIG. 20–23.—Young schizonts showing diffusely stained orthochromatic nuclei, heavily stained metachromatic cytoplasm and metachromatic granules.

FIG. 24.—Portion of schizont with well-stained orthochromatic nuclei, intense cytoplasmic stain obscuring granules (parasite distorted and from periphery of smear).

FIG. 25.—Mature schizont, merozoites with pale orthochromatic nuclei, metachromatic granule and small amount of metachromatic cytoplasm.

FIG. 26.—Uninucleate erythrocytic parasite with single metachromatic granule, parasite nucleus essentially unstained, host nucleus orthochromatic.

FIG. 27.—Uninucleate erythrocytic parasite with two metachromatic granules.

FIG. 28–29.—Schizonts, one with no apparent granules.

FIG. 30.—Multiple infection of cell.

FIG. 31.—Segmenting form.

n.e.—nucleus of capillary endothelial cell.

m.g.—metachromatic granule.

e.n.—erythrocyte nucleus.

All drawings were made by E. Bohlman Patterson with the aid of a camera lucida at ×3000.

able appearance with this technique. Female gametocytes (fig. 20–22, plate III) usually exhibit one large diffuse pink area with one or more additional light, bar-shaped or rounded pink masses nearby or connected to it. Male gametocytes usually have one fairly prominent pink mass of rounded irregular shape and as many as five to six smaller separate masses very lightly stained (note that these are intensified in fig. 23–25, plate III).

Slides of *Plasmodium lophurae* treated as above did not differ markedly in the appearance of individual nuclei from *P. gallinaceum*. *P. vivax*, stained by the Feulgen method after pigment was removed with methyl-chloroform, showed no stages that stained as intensely as those of the avian forms. Again it was possible to see most clearly the nuclei of the mature segmenters, late schizonts, and, in a few instances, very early trophozoites. Nuclei of later trophozoites, early schizonts, and gametocytes could not be seen.

With wet-fixed material followed by toluidine blue O or methyl green-pyronin staining, the nuclei of *P. vivax* closely resemble those of the avian forms (plate IV). As in the other species, the nuclei are orthochromatic with toluidine blue O, greenish with methyl green-pyronin, and all stain is readily removed by alcoholic dehydration. Again there is a great contrast between the morphology of wet-fixed preparations and air-dried films. Typical ring stages with large vacuole-like areas are missing, except at the periphery of some smears, where distortion is greatest. The vacuole, when seen, is small, and most of this area appears to be occupied by the diffusely staining nucleus. The sequence of staining intensity varying with nuclear size is also found in this species. The nuclei of mature segmenters, which stain most darkly, and those of new trophozoites are readily seen. Older trophozoites and young schizonts have lightly stained nuclei. Among the least easily seen nuclei, comparable to those of gametocytes in intensity of staining, are those of the sporozoites. Sporozoites in several stages of development, uninucleate, binucleate and quadrinucleate, were present in all the salivary gland preparations studied. In none of the preparations using the Feulgen technique could the latter be positively identified, and in toluidine blue O and methyl green-pyronin stained material they were extremely light and vacuole-like in appearance (see fig. 17–23 and 32–34, plate IV).

Changes in the host nuclei are to be seen in the avian forms. Parasitized capillary endothelial cell nuclei are often more lightly stained than those of unparasitized cells following the Feulgen technique. In some cells the nuclei become pyknotic and then stain more heavily. In the brain, other cells surrounding the areas infected also exhibit changes which have been attributed to anoxic degeneration.[21] Although not obvious in erythrocytes infected with small parasites, in later stages these nuclei also become pyknotic and are hyperchromatic with toluidine blue O, methyl green-pyronin, and Giemsa, but are more diffusely stained with the Feulgen technique. The use of other fixation methods, particularly those involving lanthanum solutions (which are of value in precipitation of nucleic acids), did not enhance the results obtained by the staining techniques reported above.

Ribosenucleic acid.—The cytoplasm of all three plasmodia stain very intensely

21. Lewert, R. M. 1948, Exoerythrocytic infection by *Plasmodium gallinaceum* in blood-infected quinine-treated chicks. Am. J. Hyg. **48**: 158–170.

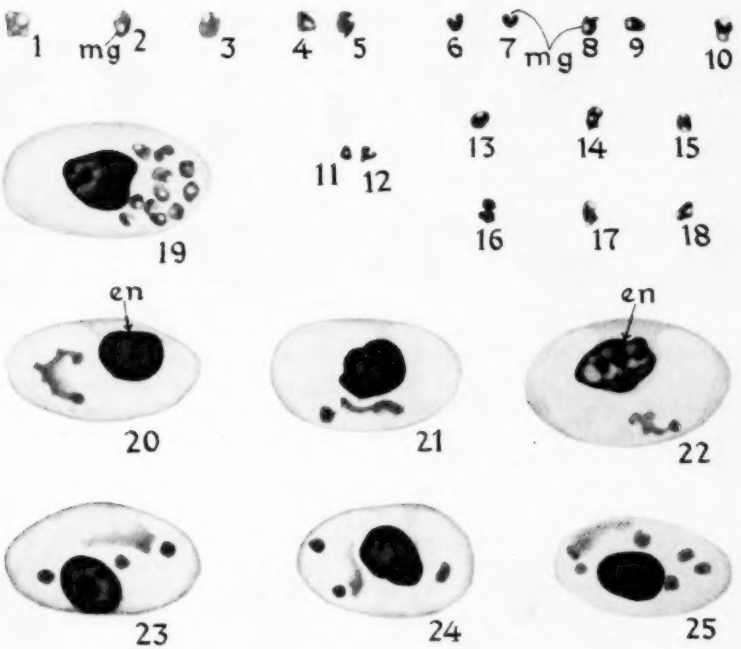

PLATE III.—Nuclei of *P. gallinaceum* following treatment with ribonuclease, dry-fixed with methyl alcohol and stained with Giemsa, pigment removed with methyl/chloroform.

FIG. 1–18.—Selected nuclei from various stages of erythrocytic parasites showing unstained area due to removal of ribosenucleic acid from the metachromatic granule.

FIG. 19.—Schizont in erythrocyte.

FIG. 20–22.—Female gametocytes in erythrocytes.

FIG. 23–25.—Male gametocytes in erythrocytes.

m.g.—area occupied by metachromatic granule.

e.n.—erythrocyte nucleus.

All drawings were made by E. Bohlman Patterson with the aid of a camera lucida at ×3000.

with either toluidine blue O or pyronin of the methyl green-pyronin stain. This relatively intense staining is characteristic of all stages with the exception of gametocytes and sporozoites. In sections of brain infected with exoerythrocytic stages of *P. gallinaceum*, the parasites stand out distinctly from the surrounding tissue following either stain. In the case of the toluidine blue O stain the cytoplasm of all stages of the three species studied is distinctly metachromatic in contrast to the orthochromatic nuclei. This cytoplasmic metachromasia is particularly striking in the avian forms where the parasite lies close to the orthochromatic host erythrocyte nucleus. The metachromasia persists following treatment with warm methyl alcohol-chloroform, or cold 5% trichloroacetic acid. However, metachromatic areas do not stain with the Hotchkiss periodic acid leucofuchsin technique or with the various modifications of the technique used, indicating that little if any mucopolysaccharide

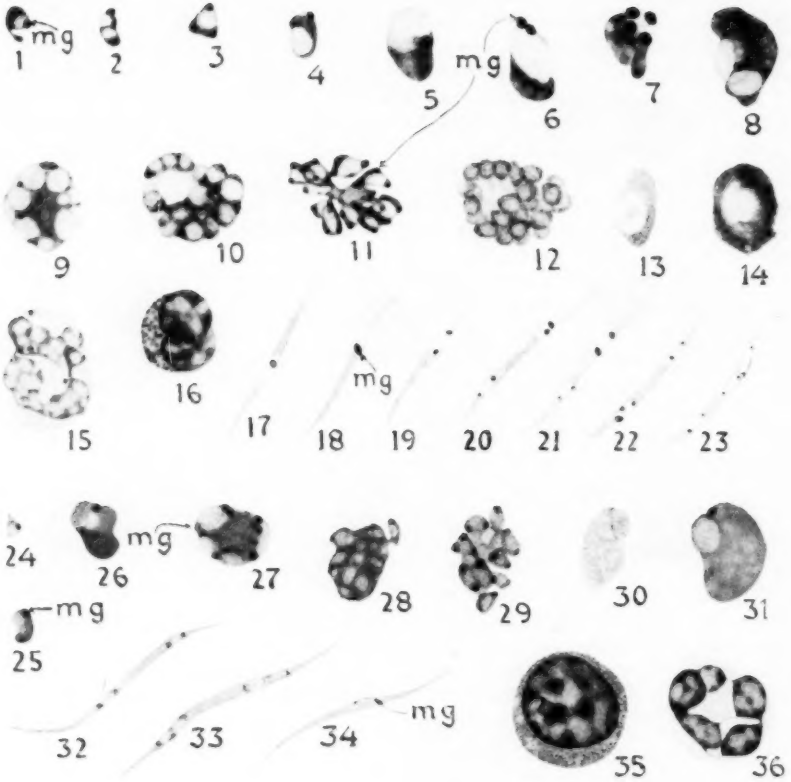

PLATE IV.—*P. vivax*.

FIG. 1–23.—Wet-fixed in FAA, pigment removed with methyl/chloroform, 0.5% toluidine blue, acetone dehydration.

FIG. 24–26.—As above, but methyl green-pyronin stain.

FIG. 1–12.—Erythrocytic series showing metachromatic cytoplasm, metachromatic granules, and pale orthochromatic nuclei.

FIG. 13–14.—Gametocytes.

FIG. 15.—Heterophil.

FIG. 16.—Small lymphocyte.

FIG. 17–23.—Various stages of sporozoites from salivary gland smear.

FIG. 24–29.—Schizogonic series showing ribosenucleic acid (pyronin-positive) in cytoplasm and in granules, and pale (methyl green-positive) nuclei containing Dna.

FIG. 30–31.—Gametocytes.

FIG. 32–33.—Sporozoites from salivary gland smear.

FIG. 35.—Lymphocyte.

FIG. 36.—Heterophil.

All drawings were made by E. Bohlman Patterson with the aid of a camera lucida at ×3000.

material is present. Cytoplasmic staining in general is reduced after treatment with desoxyribonuclease for 12 hours and is also reduced by exposure to veronal or phosphate buffers pH 7.3 to 7.5, and even warm distilled water for 12 hours will reduce the basophilia to a certain limited extent. All metachromatic cytoplasmic staining is completely removed in less than one hour by treatment with ribonuclease at 37 C. Although most of the slides stained with toluidine blue O were stained at concentrations which are known to give false metachromasia, it is believed that the plasmodial cytoplasm exhibits true metachromasia. At room temperature, slides fixed in methyl alcohol or FAA and exposed to concentrations of toluidine blue O from 0.005 to 0.05% as wet mounts show metachromatic parasite cytoplasm. After heating to 70 to 90 C, the metachromasia is retained by the parasite cytoplasm while the host nuclei remain orthochromatic.

The most striking feature of the plasmodia with respect to ribosenucleic acid is the concentration of this substance in intensely staining metachromatic granules. These are most readily demonstrated by staining with 0.5% toluidine blue O followed by rapid alcoholic dehydration. The granules are usually found at or in the periphery of the nucleus, in many instances appearing to be juxtanuclear in position. In some cases they appear to be well within the nucleus itself. These concentrations of ribosenucleic acid are found in the smallest forms of the avian parasites, both erythrocytic and exoerythrocytic (see fig. 9–31, plate II). In *P. vivax* the ribosenucleic acid concentrations are also present but are always more diffusely stained and give the appearance of less discrete structures than those found in avian forms (see fig. in plate IV). As in the avian forms these granules are also pyroninpositive. When living, the granules fluoresce as brilliant green dots following treatment with acridine O, in contrast to the almost colorless nucleus and the less deeply yellow-green fluorescent cytoplasm. They may also be observed in the living condition as dark granules under the phase microscope in avian forms as well as in *P. vivax*. The staining of the granules, as well as the cytoplasmic metachromasia, is completely eliminated by previous treatment with ribonuclease. In the flattened and somewhat distorted nuclei found in air-dried smears, the granules appear somewhat more intensely stained than the nuclei following Giemsa. As previously mentioned, nuclei in similarly prepared smears treated with ribonuclease often appear notched or perforated due to the removal of the ribosenucleic acid portion of these structures (see fig. 1–20, plate III).

As seen following the toluidine blue O stain, these distinct purple granules are to be found in the youngest parasites as soon as they have penetrated erythrocytes or capillary endothelial cells. In the smallest parasites they are 0.5 μ or slightly less in diameter, and are usually at or near the periphery of the relatively pale vacuole-like area containing the nucleus. As the parasite cytoplasm increases, the granules increase in diameter to as much as 1.0 μ. Prior to the division of the nucleus the granule divides and various stages can be found from those with a small double granule to those with granules at opposite sides of the nuclear sphere (see fig. 9–15, plate II). When the nucleus divides, and prior to new cytoplasmic growth, stages may be seen where the intensity of staining of the granules and the cytoplasm is more diffuse. At one stage in division the granules can no longer be seen (see fig. 24, 28 & 29, plate II).

In all other stages, one or two granules are present per nuclear mass. The variations in the intensity of staining of the cytoplasm give the impression of a cyclical change in concentration of the cytoplasmic ribonucleic acid. In newly formed merozoites there is very little cytoplasm, and that which is present is lightly stained and only slightly metachromatic (see fig. 25 & 31, plate II). As growth begins, the cytoplasmic staining becomes more intense and more noticeably metachromatic, in some instances obscuring the granule. When nuclei divide, the intensity of cytoplasmic staining appears to be decreased just prior to the reappearance of the metachromatic granules.

Few gametocytes were observed or could be positively identified. The cytoplasm of the female gametocytes of *P. gallinaceum* stained very faintly with either toluidine blue O or methyl green-pyronin in comparison to the other stages on the same slide. In these gametocytes a single metachromatic granule could be seen. A few stages tentatively identified as male gametocytes had a faintly staining cytoplasm and as many as eight large metachromatic granules. The cytoplasm of sporozoites also stains much lighter than that of any of the stages in the erythrocytic cycle. In sporozoites, one to four metachromatic or strongly pyronin-positive granules are to be seen. These retain the same relationship to the extremely pale spherical to ovoid nuclei as is seen in other stages of the parasite. In a few instances sporozoites were seen with granules which did not correspond exactly to the normal picture, just as in the blood preparations stages were occasionally found that could not be positively identified.

The presence of relatively large amounts of ribosenucleic acid in the cytoplasm of plasmodia may also be shown by ultraviolet absorption. Erythrocytic and exoerythrocytic stages of *P. gallinaceum* were fixed by various methods including FAA, methyl alcohol, and lanthanum mixtures, and observed at 2650 Å by the use of a fluorescent screen. Very young parasites could be observed only with difficulty, but older and larger parasites were readily visible. The intensity of cytoplasmic absorption varies from stage to stage possibly with reference to cytoplasmic growth between nuclear divisions. In some instances the parasite is as strongly absorbing at 2650 Å as is the host nucleus. However, the nuclear areas of the parasite appear, in contrast, as vacuoles, and were never seen to absorb as strongly, as the surrounding cytoplasm. Further results of a more quantitative nature will be reported in another paper.

According to some investigations[22,23] there is a correlation between alkaline phosphatase content, nucleic acid content, and a high rate of protein synthesis. Evidence has been presented that indicates that ribosenucleic acid and alkaline phosphatase are bound together so that in some cells studied they have a parallel distribution. Exoerythrocytic stages of *P. gallinaceum* were stained for alkaline phosphatase, using the method of Gomori.[15] The distribution of the enzyme as shown in figures 9 to 14, plate I, roughly agrees with the areas of high ribosenucleic acid content. However, many parasites were either very lightly stained or unstained. A few showed color development in an erratic distribution in the parasite as seen in figure 15, plate I.

22. Davidson, J. N. 1950, The Biochemistry of the Nucleic Acids, Wiley.
23. Brachet, J. 1947, Biochemical and physiological interrelations between nucleus and cytoplasm during early development. Growth, **7**: 309–324.

P fractions of brain infected with P. gallinaceum

The results of phosphorus determinations on normal and heavily infected brain are presented in table 1, and are expressed as mg of P per 100 g wet weight of fresh tissue. The figures given, with the exception of those for the inorganic fraction of the cold acid soluble P, are means with standard deviations for 8 normal and 8 infected chicks. The birds ranged in weight from 175 to 250 g, and all infected chicks were in terminal stages of the exoerythrocytic infection.

The total nucleic acid P found in the brain of the young normal birds is of approximately the same order of magnitude as that reported for mammals, i.e., 39 mg P/100 g wet weight of chick brain as compared to an average reported by various authors[24] of about 40 mg P/100 g wet weight of rat brain. However, the R/D ratio is higher in these determinations (3.5) than those reported for rat brain (2.2).

TABLE 1.—*Comparison of P fractions in normal brain and brain of young chicks heavily infected with exoerythrocytic stages of* P. gallinaceum.

	Normal (8 birds)	Infected (8 birds)
Total P (measured)	289 ± 7.6*	287 ±11.3
Total P (by addition)	298.8	297.8
Total nucleic acid P	38.6± 4.3	29.2± 3.4
Ribosenucleic acid P	30.6± 3.5	23.8± 4.4
Desoxyribosenucleic acid P	8.7± 1.7	7.0± 0.8
Ratio R/D	3.5	3.4
Protein P	8.9± 0.3	8.0± 0.8
Cold acid soluble P	84.5± 4.6	75.4± 6.0
Inorganic (Mg pptl.) P	60%†	58%‡
Lipid P	135.5±17.6	154.4±10.4

Figures given in terms of mg P/100 g wet weight of fresh tissue.
* Mean with standard deviation.
† Average of three determinations only.
‡ Average of two determinations only.

The changes in the various P fractions in the infected birds are not as great as one is led to expect from the staining reactions. Instead of the anticipated increase in nucleic acid P, there is a significant decrease in total nucleic acid. Most of this change is accounted for by a decrease of the ribosenucleic acid fraction. A decrease from normal is also found in the cold acid soluble fraction, although the proportion of inorganic to organic P in this fraction was essentially unchanged in the samples tested. The only significant increase is found in lipid phosphorus, the alcohol and ether soluble fraction. As will be seen later, very significant increases in this fraction are found in infected erythrocytes.

P determinations of erythrocyte fractions

The results of these determinations, presented in tables 2, 3, 4, and 5, are expressed as μg P/1$\times 10^9$ erythrocytes. This figure was chosen as a matter of convenience in the calculations. Errors due to the inclusion of plasma were eliminated by washing the citrated cells in cold saline, and those errors due to the inclusion of leucocytes were avoided by removing the buffy coat at each washing of the cells. Failure to remove leucocytes resulted in slightly higher values, giving about a 5% increase in total P measured. Erythrocyte and parasite counts were made after washing the cells and removing the leucocytes. In order to avoid the effect of reticulocytes on these determinations, no analyses of samples in which more than 2% of the cells were of the immature type are included in the tables.

The effects of erythrocytic infection on the P levels of erythrocyte fractions in young chicks are presented in table 2. All birds in this series were approximately 250 g in weight. The normal figures are given as means with standard deviations for 5 birds. The 5 parasitized blood samples were taken from

24. Davidson, J. N. 1947, Some factors influencing the nucleic acid content of cells and tissues. Cold Spring Harbor Symp., Quant. Biol. **12**: 50–59.

TABLE 2.—*Comparison of the P fractions of normal erythrocytes and those of selected* P. gallinaceum *infections in 250 g birds.*

	5 normal	(1)	(2)	(3)	(4)	(5)
Total P (measured)	347 ±11	348	440	460	414	441
Total P (by addition)	343 ± 5.3	338	427	464	405	443
Rna P	5 ± 2.3	13	45	53	30	73
Dna P	191 ± 8.8	180	205	222	205	191
Protein P	12.5± 1.6	13	12	12	15	7
Cold TCA P	101 ± 6.5	104	106	104	102	98
Lipid P	28 ± 3.4	27	60	74	53	74
Parasites per 1×10^4 rbc		2180	6720	7150	9490	11,440
Percent parasitemia		20	56	66	73	88
Percent trophozoites		78	62	17	79	52
Percent schizonts		21	37	73	14	41
Percent segmenters		1	1	9	4	6
Percent doubtful & gametocytes				1	3	1
Percent reticulocytes			Less than 1 in all samples			

Results are expressed as rP/1×10^9 rbc.

chicks during the first few days of infection prior to the development of large numbers of reticulocytes. In this series of individual infections relatively large increases in total P are found, the greatest being more than 30% above the normal mean. The increases are not equally distributed throughout the various fractions but are most apparent in lipid P, which more than doubles, and in ribosenucleic acid P (Rna P), which increases 13-fold. The desoxyribosenucleic acid P (Dna P) shows little increase over the normal except in the case of bird 3. Neither protein P nor cold acid soluble P (cold TCAP) show significant changes in this series of infections.

It is obvious from examination of the tables that the changes in P values are not directly proportional to the percentage of cells parasitized or to the total number of parasites. The most consistent relationship between degree of infection and P increase occurs in the Rna P fraction. The increase in Rna P is not great in infections in which there is a high percentage of small trophozoites, as in animal 4. In this animal all P fractions are less than might be anticipated if animal 3 were used for comparison. This lighter infection, with large trophozoites and high percentage of dividing forms, has higher P values in every fraction. It is further interesting to note that in the lightest infection (20% parasitemia, bird 1) presented in this table all of the P fractions, with the exception of the Rna P, are in or below the range of the normal mean. This indicates an apparent depletion of the host cell's phosphorus and is more evident in the determinations made on light infections in older birds to be dis-

TABLE 3.—*Comparison of the P fractions of normal erythrocytes and those of individual infections in 2500 g birds followed daily (bird L-3-1, female).*

	4 normal	Day 1	Day 2	Day 3	Day 4
Total P (measured)	390 ±3.7	395	387	338	493
Total P (by addition)	387 ±2.4	387	378	335	502
Rna P	13 ±2.0	13.5	30.5	15	68
Dna P	208 ±9.0	209	204	173	216
Protein P	14.6±2.8	18	12.4	20	19
Cold TCA P	109 ±3.5	106	106	94	128
Percent inorganic P	32	35	33	27	20
Lipid P	42 ±2.8	40	29	28	73
Parasites per 1×10^4 rbc			694	2889	7375
Percent parasitemia			6.8	26.5	59
Percent trophozoites			98	87	49
Percent schizonts			2	10	48
Percent segmenters				3	2
Percent doubtful & gametocytes					1
Percent reticulocytes			Less than 1 in all samples		

cussed in the next series of experiments.

The progressive changes in nucleic acid and other phosphorus-containing fractions of infected erythrocytes were also investigated. Early stages of the infection were studied in birds older than those used in the previous determinations. Normal blood samples were taken from 2500 g birds on the day of infection, and samples were taken at 24-hour intervals thereafter during the initial rise of the parasitemia. The use of these larger birds permitted daily bleeding for 4 or 5 days without a marked increase in the number of reticulocytes. The results of phosphorus determinations on these samples are presented in tables 3, 4, and 5. The means with standard deviations for 4 normal birds of the same age and weight are included in table 3.

Total P and all other P fractions in these mature, normal birds are higher than in the small, normal chicks of table 2. The amount of desoxyribosenucleic acid in these erythrocytes is of the same order of magnitude as that reported by other authors who made phosphorus determinations on isolated fowl erythrocyte nuclei.[25–27] They are considerably higher than the results obtained by Kurnick using an absorption technique following staining. Converting the P values to mg of nucleic acid to correspond with the results of other authors, the figures compare as shown in table 6.

TABLE 4.—*Comparison of the P fractions of normal erythrocytes and those of individual infections in 2500 g birds followed daily (bird L-10, female).*

	Day 1	Day 2	Day 3	Day 4
Total P (measured)	386	352	485	551
Total P (by addition)	385	341	495	541
Rna P	15	13	26	61
Dna P	197	182	217	239
Protein P	15	12	13	13
Cold TCA P	114	99	186	141
Percent inorganic P	24	26	18	14
Lipid P	46	35	53	77
Parasites per 1×10^6 rbc		900	1438	13,464
Percent parasitemia		9	13	77
Percent trophozoites		99	11	69
Percent schizonts		1	82	30
Percent segmenters			6	1
Percent doubtful & gametocytes			1	
Percent reticulocytes		0	0.1	2.0

TABLE 6.—*Conversion of P values to mg of nucleic acid, to correspond with the results of other authors.*

Author	Mg $\times 10^9$ nuclei	
	Dna P	Rna P
Mirsky and Ris	2.34	
Davidson and McIndoe	2.62	0.09
Kurnick	1.6 −1.8	
Present work	2.24±0.08	0.13±0.02

TABLE 5.—*Comparison of the P fractions of normal erythrocytes and those of individual infections in 2500 g birds followed daily (bird L-11, male).*

	Day 1	Day 2	Day 3	Day 4	Day 5
Total P (measured)	389	376	419	416	466
Total P (by addition)	387	387	423	438	453
Rna P	12	21	13	18	34
Dna P	210	215	249	240	228
Protein P	11	17	16	16	13
Cold TCA P	107	104	115	109	121
Percent inorganic P	38	44	41	33	12
Lipid P	40	33	39	42	52
Parasites per 1×10^6 rbc		740	4142	6420	11,657
Percent parasitemia		7.4	35	54	67
Percent trophozoites		89	95	63	82
Percent schizonts		11	4	32	17
Percent segmenters			1	5	1
Percent doubtful & gametocytes					
Percent reticulocytes		0	0	0	2

25. Kurnick, N. B. 1950, The quantitative estimation of desoxyribonucleic acid based on Methyl Green staining. Exp. Cell. Res. **1**: 151–157.

26. Mirsky, A. E. and Ris, H. 1949, Variable and constant components of chromosomes. Nature, **163**: 666–667.

27. Davidson, J. N. and McIndoe, W. M. 1949, Phosphorus compounds in the cell nucleus. Biochem. J. **45**: Proc., 16.

Each of the infections in the older birds shows a depletion of the P of the host cell, a condition suggested by the lightest infection studied in younger birds (table 2, bird 1). Decreases below the mean of the normals are found in all P fractions with the exception of protein P and ribosenucleic acid P. In each case the decreases are perceptible when the infection is relatively light (900 to 3000 parasites per 10,000 rbc) and when the percentage of small trophozoites is high (table 2, bird 1; table 3, days 2 and 3; table 4, day 2; table 5, day 2). As the birds become more heavily infected (over 3000 parasites per 10,000 rbc) there are general increases in all P fractions. As in the younger series of birds, the increases are not directly proportional to the degree of parasitemia. The maximum increase in total P in this series (table 4, day 4) is greater than that found in the younger birds (40%). In this series, as well as in the previous one, the increase in parasite Dna P is masked by the depletion of the P of the host nuclei, and the greatest increase over the normal mean is about 11% (table 4, day 4). The increase in ribosenucleic acid P, as shown in tables 3, 4, and 5, is not as great as that seen in table 2. However, in other determinations in which some P fractions were not measured and which are therefore not presented in the tables, increases up to 10 times greater than the normal mean were seen. Also, larger increases in phospholipids, up to 3 times the normal mean, were found in other incomplete determinations. In these older birds, the lipid P increase was usually less than this figure and is particularly low in one infection (table 5).

Some increase with heavy infection is found in the cold, acid soluble fraction. As the infection progresses, there is both a relative and an absolute decrease in inorganic phosphorus within this fraction, with a corresponding increase of the organic portion of the fraction.

DISCUSSION

Caspersson and his coworkers[28] maintain that ribosenucleic acid is associated directly with, or is essential for, the production of the main bulk of cytoplasmic protein in cell growth. The ribosenucleic acid-containing nucleolus is interpreted as being a controlling center or at least an active structure in this process of cytoplasmic protein synthesis. Many of the cells studied which are engaged in intensive protein production have a large, prominent nucleolus and the cell cytoplasm is basophilic owing to the abundance of ribosenucleic acid.[29] In such cells there is usually a high alkaline phosphatase content. Whether or not this interpretation is correct, it is not surprising to find in the rapidly growing plasmodia concentrations of ribosenucleic acid and a ribosenucleic acid cycle that changes with growth and nuclear division in a manner similar to that reported for other actively growing and dividing cells. From the standpoint of ribosenucleic acid content, the juxtanuclear metachromatic granules of plasmodia resemble the nucleoli of other cells. Their fluctuation in size and in intensity of staining during growth and between nuclear divisions seem to parallel the changes in concentration of ribosenucleic acid in the cytoplasm. They are absent or very diffusely stained at the time of nuclear division and are most apparent during cytoplasmic growth between divisions. An additional parallel is found in the distri-

28. Caspersson, T. O. 1950, Cell Growth and Cell Function: a Cytochemical Study, W. W. Norton & Co.

29. Bing, J., Fagraeus, A. and Thorell, B. 1945, Studies on nucleic acid metabolism in plasma cells. Acta physiol. Scandinav. **10**: 282–294.

bution of alkaline phosphatase which is located primarily at the sites of ribosenucleic acid concentration.

Various authors have described structures in the cytoplasm or associated with the nucleus of plasmodia which are probably the same as those identified here as containing ribosenucleic acid. However, since their relationship to the nucleus is not always well-preserved in air-dried smears, these structures have been interpreted in a variety of ways. Their presence undoubtedly accounts for the reports of "volutin" granules in various stages of the parasite, including sporozoites.[30] With Giemsa's stain, depending on the technique, they often appear a slightly darker red to purple as compared to the color of the main mass of the nucleus. These are probably the structures described by various authors[9,31–33] as accessory nuclei or blepharoplasts, so as to include the plasmodia in Hartmann's binucleate theory of protozoan classification. That these structures are not artifacts produced by fixation or staining is apparent, since they can be seen in living cells with the phase microscope. Also, the structures containing these concentrations of ribosenucleic acid are well shown in the living parasites following staining with fluorochromes, as described previously. Acridines are known to be bound by ribosenucleic acid[34] and after staining with acridine O, structures containing ribosenucleic acid fluoresce strongly in the plasmodia. The variation in intensity of this fluorescence agrees well with the distribution of and variation in Rna of the parasites, as seen by other methods of staining as well as by ultraviolet absorption of fixed material at the absorption maximum for nucleic acids.

Considering the activity and the invariable association of the Rna-containing structures with the nucleus, it does not seem reasonable to compare them to "storage granules," "volutin," or accessory nuclei. It is suggested that they are intimately concerned with nucleic acid synthesis and may be comparable to the nucleolus of other cells.

In spite of the impression given by staining techniques of apparent increases in Rna in the parasitized brain, no increases were found in the quantitative phosphorus determinations. This result was not expected, since each of the infected brains studied was estimated to have at least 10,000 parasites per cmm. The normal brain has relatively large amounts of Rna which might be utilized by the parasites, as it has been shown that plasmodia have ribonuclease activity.[35] The possibility that plasmodia can utilize directly such materials as ribosenucleic acid, nucleotides and nucleosides has been considered. However, large amounts of these substances administered intraperitoneally to infected chicks has no effect on the course of exoerythrocytic infection.[36] Decreases in brain Rna might also be due to the anoxia which results from heavy infection of the brain capillaries. Anoxic areas, with degenerating nerve

30. Mudrow, L. 1942, Zur Sporozoitementwicklung verscheidener Vogelmalariaarten. Riv. di malariol. **21**: 382–385.
31. Flu, P. C. 1908, Untersuchungen über Affenmalaria. Arch. f. Protistenk. **12**: 323–336.
32. Mayer, M. 1908, Über Malariaparasiten bei Affen. Arch. f. Protistenk. **12**: 314–322.
33. Swellengrebel, N. H. 1915, Over de schizogonie van de Quartanparasiet (P. malariae). Geneesk. tijdschr. v. Nederl.-Indië. **55**: 1.
34. Irvin, J. L., Irvin, E. M. and Parker, F. S. 1949, The interaction of antimalarials with nucleic acids. Science, **110**: 426–428.
35. Miller, Z. B. and Kozloff, L. M. 1947, The ribonuclease activity of normal and parasitized chick erythrocytes. J. Biol. Chem. **170**: 105–120.
36. Kozloff, L. A. and Lewert, R. M. 1945, Unpublished data.

cells, have been previously described in this stage of the infection.[21] Under these conditions, the cell may lose ribose nucleotides and, in addition, nerve cells may lose nucleotides when the animals are motorially exhausted from intense muscular activity,[37] as would be the case in terminal stages of the exoerythrocytic infection.

Only small amounts of Rna are found in the normal avian erythrocyte, and increases due to parasite Rna are more easily demonstrated than in the brain. In those infections in which one finds approximately one parasite per erythrocyte, as much as a tenfold increase above normal is found in the Rna. In the previous determinations of nucleic acid in *P. knowlesi*,[10] no attempt was made to separate nucleic acid P into its components, and the major increase in nucleic acid P was assumed to be due to the increase in nuclear nucleic acid, the desoxyribosenucleic acid. The results from these determinations in an avian plasmodium show that a large portion of the increase is due to ribosenucleic acid. However, the amount of increase in Dna is masked in these determinations since there is a degradation of host Dna. This decrease in host nuclear material is greater at first, when the parasite is small and uninucleate, than the synthesis of Dna by the parasite. Later, when the parasite is larger and multinucleate, the synthesis of Dna by the parasite plus the residual Dna in the host nucleus gives an increase above the normal figure.

The blood analyses also show decreases in other phosphorus-containing fractions within the parasitized avian cell. These decreases indicate parallels between this form and the metabolism of simian forms. In the latter, the necessity for phosphorus in vitro and in vivo has been well demonstrated.[38] In these investigations of mammalian forms, increases in phospholipids up to two to four times normal have been found. In the avian forms reported here, over three times the normal figure is found when there is an average of approximately one parasite per cell. The amount of lipid synthesized is probably much greater than this, since no attempt was made to estimate fatty acid synthesis.

Another indication of the possible utilization of phosphorus-containing compounds is to be seen in the changes in acid-soluble P. There is some indication that this total fraction increases slightly in heavily infected cells in the older birds studied. However, there is a progressive decrease in the inorganic portion of this fraction with a corresponding increase in the organic phosphorus. The determinations made of the protein P fraction were quite variable and did not show significant increases in the infected cells and tissues. Some increase might be anticipated since Groman[39] demonstrated that although total nitrogen of infected cells did not increase, some increases in amino nitrogen are found in parasitized cells.

SUMMARY

The presence and distribution of ribose and desoxyribose nucleic acids in *Plasmodium gallinaceum*, *Plasmodium lophurae*, and *Plasmodium vivax* have been described. Staining techniques indicate that there is a marked ribose nu-

37. Hyden, H. 1943, Protein metabolism in the nerve cell during growth and function. Acta physiol. Scandinav. **6**: Suppl. 17.

38. McKee, R. W., Ormsbee, R. A., Anfinsen, C. B., Geiman, Q. M. and Ball, E. G. 1946, Studies on malarial parasites. VI. The chemistry and metabolism of normal and parasitized (*Plasmodium knowlesi*) monkey blood. J. Exper. Med. **84**: 569–582.

39. Groman, N. B. 1951, Dynamic aspects of the Nitrogen metabolism of *Plasmodium gallinaceum*. J. Infect. Dis. **88**: 126–150.

cleic acid cycle in the parasite. This is associated with or accompanied by the activity of a juxtanuclear structure which behaves in a manner similar to that of a nucleolus. This structure is Feulgen-negative, metachromatic following toluidine blue, pyronin-positive, unaffected by desoxyribonuclease, but completely unstained following treatment with ribonuclease. One of these metachromatic granules is associated with or in the border of each nondividing nucleus. They divide prior to nuclear division. These structures may also be demonstrated in the living plasmodia by phase microscopy or by vital staining following the intravenous injection of the host with fluorochromes. Their activity may be comparable to the activity described for the nucleolus of other rapidly growing cells where this is interpreted by some authors as being a regulatory center for protein synthesis in cell growth between nuclear divisions.

Although preliminary study has shown that in the ultraviolet microscope at the absorption maximum for nucleic acids the plasmodial nucleus is relatively nonabsorbing, desoxyribose nucleic acid is present. This can be demonstrated by the Feulgen reaction, methyl green of the methyl green-pyronin technique, and by their orthochromatic staining with toluidine blue after removal of ribose nucleic acid with ribonuclease. The nuclei progressively decrease in size and increase in the intensity of their staining in the schizogonic series. In wet-fixed preparations they appear almost invariably to be spheres with a homogeneous distribution of Dna with no granules or structure comparable to those seen in other cells. In some stages interpreted as being dividing nuclei, elongate forms are seen with their central axes less intensely stained. Except in the most mature stages of the parasite with small nuclei, the intensity of staining is never comparable to that of the host nuclei.

Other cytochemical techniques show that alkaline phosphatase is present in the plasmodium and that its distribution closely parallels that of ribose nucleic acid. It was not possible to detect any glycoprotein in any of the stages studied using periodic acid leucofuchsin techniques, and in spite of the production by the parasites of large amounts of phospholipids, plasmal reactions were negative.

Quantitative studies of nucleic acids and other phosphorus-containing fractions of cells and tissues have been made. Brain from chicks in terminal stages of the exoerythrocytic infection was fractionated and analyzed for phosphorus and compared with a series of uninfected animals. Unexpectedly, no significant increase in total phosphorus, nucleic acid phosphorus, cold acid soluble phosphorus or protein phosphorus was found. Some increases in lipid phosphorus could be demonstrated. The fact that nucleic acid phosphorus did not increase in the heavily infected brain is interpreted as being due to a depletion of the considerable quantities of host cell nucleic acid normally found in the brain.

Phosphorus analyses of erythrocytes relatively heavily infected with *P. gallinaceum* from chicks in various stages of the infection show that there are decided increases in total phosphorus. Most of this increase is due to increases in ribosenucleic acid phosphorus and lipid phosphorus although there are some increases in cold, acid soluble phosphorus and desoxyribosenucleic acid phosphorus. Protein phosphorus remains essentially unchanged. The increases in the various fractions cannot be directly correlated with the degree of parasitemia or the number of parasites since the changes are also related

to the predominant stage of development present at the time blood was removed for analysis. Infections in older birds were followed by daily analyses. In relatively light infections with a large percentage of rapidly growing trophozoites there is evidence of considerable depletion of host material and values for total phosphorus, desoxyribosenucleic acid phosphorus, and lipid phosphorus are considerably lower than normal. Ribosenucleic acid phosphorus increases can be demonstrated since the host erythrocyte has very little of this substance. The initial decreases in the various phosphorus fractions mask the actual increase due to parasitism early in the infection. Later in the infection as in the young birds, significant increases in all fractions are found with the exception of protein phosphorus. As the infection progresses there are changes in the cold acid soluble fraction as well. The total of this fraction increases and the proportion of the total consisting of organic phosphorus increases with a corresponding decrease in inorganic phosphorus.

STUDIES OF DENGUE FEVER VIRUS IN THE CAVE BAT (*MYOTUS LUCIFUGUS*)

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The dengue fever virus strain (Hawaii mouse-adapted) used for this study was secured from the American Type Culture Collection of Washington, D.C. This strain was furnished to that group by Dr. A. B. Sabin of Children's Hospital, Cincinatti, Ohio. Following its isolation in 1944, the virus was maintained for several serial passages in human volunteers and was propagated in mice by intracerebral passage (Sabin et al, 1945). Lyophilized mouse brain of the 114th intracerebral mouse passage was received at this Maryland laboratory to initiate the current experiment. In its present form the virus is highly pathogenic when injected intracerebrally into mice but is not pathogenic for hamsters, guinea pigs, rabbits or cotton rats, as reported by Sabin (1945). Mice exhibit symptoms of central nervous system involvement 5 to 6 days after inocultation with a 10% suspension.

Because the cave bat (*Myotus lucifugus*) has been shown to be highly susceptible to Newcastle disease virus (Reagan et al, 1950), experiments were conducted in an attempt to transmit the dengue fever virus (Hawaii mouse-adapted strain) to this mammal. Lyophilized mouse brain of the 114th intracerebral mouse passage was diluted to a 10% suspension with sterile distilled water. Each of eight 3-week-old Swiss albino mice was inoculated intracerebrally with 0.03 ml of this suspension. On the 5th day post inoculation the mice showed symptoms of central nervous system involvement. When symptoms appeared the mice were sacrificed, and the brains were removed aseptically. A pool of these infected mouse brains was ground with alundum and diluted to a 10% suspension with physiological saline. This suspension was then subjected to centrifugation for 3 minutes in an angle centrifuge at 1000 r.p.m. Three-hundredths ml of the supernatant was inoculated into the right frontal lobe of each of 8 cave bats. Within 3 to 11 days, the bats showed symptoms of central nervous system involvement such as irritability and paralysis of hind legs. When these nervous symptoms developed in the bats, the brains were removed aseptically, ground with alundum, and diluted to a 10% suspension with physiological saline. Amounts of 0.03 ml were injected into each bat of the succeeding serial passage throughout 6 passages. Data of passages are shown in table 1. Each virus suspension that was injected intracerebrally in bats was also injected intracerebrally in 3-week-old Swiss albino mice to see if the virus was still active for the original mouse host. The results of the intracerebral injections of bat-propagated dengue virus into mice are recorded in table 2.

TABLE 1.—*Bat passage of a strain of dengue fever virus (Hawaii mouse-adapted).*

Passage no.	Number of bats inoculated	Number of bats paralyzed, moribund or dead	Minimum to maximum incubation period (days)
1	8	3	3–11
2	10	4	6–12
3	10	6	10–12
4	8	2	9–13
5	6	2	13–17
6	4	4	14–15

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TABLE 2.—*Bat-passaged virus material injected intracerebrally in three-week-old Swiss albino mice.*

Passage no.	Number of mice inoculated	Number of mice paralyzed, moribund or dead	Maximum to maximum incubation period (days)
1	6	6	9–11
2	6	2	13–16
3	6	2	12
4	8	2	13
5	6	3	12–14
6	6	2	10–12

A virus neutralization test was conducted using brain suspension of the 6th intracerebral bat passage with specific rabbit dengue antiserum and normal rabbit serum, using mice as the test host. Specific immune serum completely neutralized the virus, while normal rabbit serum did not. The virus titrated 10^{-3} according to the Reed-Muench (1938) method of calculation.

SUMMARY

The cave bat (*Myotus lucifugus*) is susceptible by intracerebral inoculation to dengue fever virus (Hawaii mouse-adapted strain). This strain was carried for six passages in the bat by intracerebral inoculation and was confirmed to be dengue fever virus by a neutralization test done by intracerebral inoculation in Swiss albino mice. Mice injected intracerebrally from each bat passage showed nervous symptoms such as found in mice injected with the original mouse passage material used to initiate the experiment.

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ACQUIRED IMMUNITY IN MICE INFECTED WITH *SCHISTOSOMATIUM DOUTHITTI*

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The immunology of schistosomiasis has interested workers since the elucidation of the life cycle of *Schistosoma japonicum* during the early part of this century. Much emphasis has been placed on the development of serological and cutaneous diagnostic tests Ozawa (1930) in a review of acquired immunity towards infections with *S. japonicum* reported the following: Acquired immunity had been found in cattle and humans from endemic areas by workers in Japan. Oxen require five years to become completely resistant to reinfection. Horses are completely resistant after initial infection and immunity was not demonstrable in experimentally infected rabbits and dogs. In his own work he noted resistance to superinfection in infected dogs treated with stibinol, but his experiments do not appear to be adequately controlled. He also found that dogs immunized with saline emulsions of worms and cercariae were more resistant to infection than controls.

Kawamura (1932) reported that dogs cured by intravenous injections of antimony salts were more resistant to reinfection than similarily treated rabbits. Reinfection was done by "smearing the shaved abdominal skin with snail's livers containing active cercariae." Animals receiving aqueous extracts of parasites and serums of infected animals withstood infection better than the controls. Development of worms in resistant hosts was retarded. Watarai (1936) studied the skin reaction in rats following infection and reinfection with *S. japonicum*. He found that a small number of cercariae in immune rats did not cause an inflammatory response, whereas a large number of cercariae produced an area of marked inflammation leading to the destruction of many cercariae in the skin.

Vogel and Minning (1949a) and Vogel (1949) in a preliminary paper reported 3 of 11 dogs infected with *S. japonicum* when challenged with killing doses of cercariae 13 to 27 months after initial infection demonstrated resistance to superinfection Monkeys developed a partial immunity to superinfection 304 to 471 days after infection with *S. japonicum* and a stronger resistance to superinfection after 420 to 589 days. One infected monkey cured by chemotherapy was resistant to superinfection 517 days after cure. Two monkeys resistant to *S. japonicum* showed partial heterologous resistance to superinfection against *Schistosoma mansoni* cercariae. Two monkeys infected with male *S. japonicum* when re-exposed with lethal numbers of cercariae evidenced resistance in one and partial immunity in the other 598 to 702 days after initial exposure. Attempts to develop a resistance in animals with extracts of dead and fresh worms and serums of in-

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fected animals were unsuccessful. The titer of the complement fixation reaction in infected monkeys did not parallel the development of resistance to superinfection. These authors are of the opinion that the principal sites where parasites are killed in resistant animals are the blood vessels of the internal organs and not the skin.

Fairley, Mackie and Jasudasan (1930a), working with a bovine schistosome, *Schistosoma spindale*, reported that rhesus monkeys demonstrated spontaneous recovery approximately 11 days after infection and an enhanced refractiveness during which schistosomulae failed to develop for several months. Thereafter, susceptibility returned and the animals could be reinfected. Guinea pigs (Fairley et al, 1930b) were very resistant to infection with this species, and exposure to thousands of cercariae resulted in no female worms developing, whereas male worms were malformed and atypical.

The possibility that man may become infected exclusively with male *Schistosoma mansoni* or *Schistosoma haematobium* was considered by Fairley (1926), Faust (1928) and Girges (1934). Girges believed that an infection of male worms in man provoked a certain degree of immunity which prevented the arrival of female worms to mate with them. According to Watts (1949), mice vaccinated with a saline extract of adult *S. mansoni* were resistant to infection with ten cercariae.

In an unpublished thesis, El-Gindy (1950) reported preliminary experiments on the immunology of *Schistosomatium douthitti*. He reported that following an initial infection in mice there was a partial immunity towards male worms in the challenging infection and a "pronounced harmful effect" on female worms.

S. douthitti was selected in the present study because it is a natural parasite of the mouse and muskrat, and an immune response might thus be easier to detect and to study. Techniques of infection and superinfection made it possible to expose mice to infections of known sex and exact numbers of cercariae. Three experiments were made. A total of 171 mice were infected with males, females, and male and females, respectively, and were then challenged with 50 cercariae of opposite or same sex at monthly intervals for 7 (exp. 3) or 4 months (exp. 1 and 2). Thirty mice were infected repeatedly 6 or 7 times at regular intervals and were challenged once with 50 cercariae 1 to 15 weeks later. A total of 32 experiments with 201 experimental and 200 control mice were made in this study. The number of worms recovered and the rate of development of the schistosomulae from the challenging infection in infected mice as contrasted to controls was the criterion for determining immunity to superinfection.

MATERIALS AND METHODS

S. douthitti, a schistosome of *Microtus pennsylavanicus*, the meadow vole, was obtained from naturally infected snails, *Lymnaea palustris* (Müller), collected from small ponds near Ann Arbor, Michigan. The parasite was maintained in laboratory-reared *Peromyscus maniculatus* initially obtained from the Laboratory of Vertebrate Biology, University of Michigan, and two species of *Lymnaea*, *L. stagnalis apressa* Say and *L. palustris* (Müller). An inbred strain of white mice, CFI (Carworth Farms strain), was used for all experiments with one exception, when *dba* mice were employed. In the beginning male and female mice were used, but later only females because they do not attack one another when caged in small groups. Lots of 50 to 100 six-week old mice weighing approximately 20 g were divided at random into an experimental and control group. These were kept in separate adjoining cages. The experimental group was initially exposed to a certain number of cercariae of known sex placed on the abdomen of the mouse or were immersed in small containers to approximate numbers of cercariae. The sex of cercariae from

individual snails was determined by preliminary infections in mice. A large infected snail which generally yielded 1500 to 3000 cercariae during one evening was used for a given experiment to insure uniform infections. Snails were isolated in small containers filled with water the afternoon of the day mice were to be exposed, and the cercariae were used one to two hours after they were shed. Cercariae from infected snails behave as do those of *S. japonicum* in that they appear in the water several hours after dark, move around for a brief period and then rise to the surface film of the water where they remain motionless. This biological characteristic makes it possible to remove a small number of cercariae from the surface film of the water with a small loop, to count them while still under a binocular microscope in the film of water of the loop and to transfer them to the clipped moistened abdomen of a mouse strapped to a small board. The infective dose usually consisted of 40 to 70 female, or male and female cercariae or 100 to 300 male cercariae. At intervals after infection, a small number of infected and control mice were selected at random from the original group of animals, and were weighed and challenged with 50 cercariae applied to the clipped abdomen. An exception was made in series 5 and 13, where 100 cercariae were used.

Resistance to reinfection was measured by the difference in the number of worms recovered in the liver and mesenteric veins and their rate of development in infected and control animals. Autopsies were performed 16 to 25 days after exposure to the challenging infection. This interval allows the schistosomulae to reach the hepatic portal system and grow enough to be easily recovered. Worms from the challenging infection may be readily separated from worms of the primary infection by their small size and incomplete sexual development. Mice were killed with ether and perfused with a solution containing 2% sodium citrate and 0.85% sodium chloride. The liver was not removed from the animal and only the hepatic vein between the liver and the heart was ligated. The hepatic portal vein was cut and the animal perfused from the dorsal aorta and the liver from the hepatic sinus. After perfusion, the intestine and liver were removed and searched for worms that might not have been removed. At first, the mesenteric veins were scanned with the aid of a binocular dissecting microscope. Later, worms were readily seen with the aid of a binocular eye-loupe when the mesenteries were teased in a petri dish placed on a white enamel pan. The spleen, liver and lungs were broken into small pieces by macerating them for two or three seconds in a semi-micro cup with a Waring blendor. This technique frees worms that may be in these organs without destroying them. All washings from the tissues and perfusion were concentrated, prior to being discarded, in a small funnel closed at the small end with a rubber tube and clamp. The concentrated material was then searched for worms. Schistosomes recovered from autopsies were examined under a binocular microscope, sexed, differentiated into worms of primary or secondary infections, counted, and fixed in cold Bouin's fluid. Fixed worms were stained in alcoholic cochoneal and mounted in pickylite. The length of the worms was measured after magnifying the worms ten times with a photographic enlarger. A line was drawn through the twisted and curved distome longitudinally and measured with a pair of dividers with points set 2 mm apart. Measurements were made within 0.5 mm limits.

Values of t and P were calculated (using the formulas of Snedecor, 1946) to determine the significance of differences in the number of worms in experimental and control animals. Differences with $P \leqq 0.05$ were considered significant.

EXPERIMENTAL RESULTS

Experiment 1A (series 1–10): mice exposed once initially to male and female cercariae and challenged with male, female, or male and female cercariae.—Ten series of mice, initially exposed to 43 to 100+ cercariae of both sexes were tested 19 to 123 days later with 100 male cercariae in series 5 and 50 female or male and female cercariae in the rest. A marked acquired immunity to superinfection was present at 35 days and continued through the period of the experiment, or 123 days (fig. 1). This conclusion is shown by differences between the percent of worms of the superinfecting dose recovered at autopsy in figure 1. The statistical validity of the differences is shown by the value of P in table 1. The data after 61 to 63 days is particularly significant. Although the samples were small, $P = 0.01$ for a superinfection with females (series 4, 6 and 7) and 0.05 for a twofold infection with males (series 5) were obtained. Furthermore, female worms of the challenging infection were stunted (table 1). Male worms were

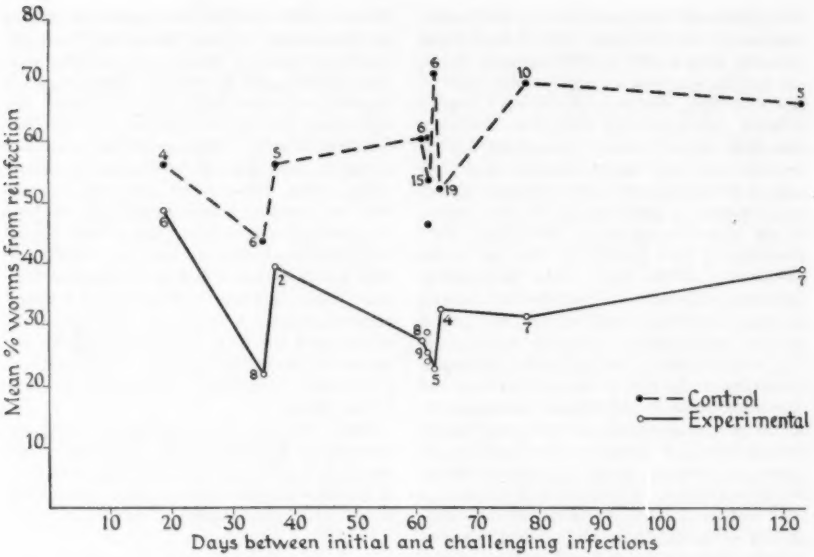

FIG. 1.—Mean percent recovery of worms from the challenging infection in mice infected with male and female worms 9 to 123 days previously.

TABLE 1.—*Experiment 1, consisting of 12 series of mice showing acquired immunity to superinfection 30 to 123 days after an initial infection with male and female cercariae.*

Exp. no.	Series	Initial exposure to both sexes: Number of cercariae	Initial exposure to both sexes: Mean worms recovered at autopsy	Challenging exposure*: Number of mice autopsied: Infected	Number of mice autopsied: Normal	Days after first exposure	Sex of worms	Percent worms recovered at autopsy: Infected	Percent worms recovered at autopsy: Normal	Percent worms recovered at autopsy: Mean difference	Percent worms recovered at autopsy: P	Mean length of worms in mm: Age of worms	Mean length of worms in mm: Infected	Mean length of worms in mm: Normal
1A	1	100+§	67.3	6	4	19	F	48.6	56.0	3.67±3.29	0.3			
	2	51	32.5	8	6	35	MF	21.8	43.3	13.3 ±2.82	<0.01	19	1.32 M	1.46
	3	100+§	62.5	2	5	37	F	39.8	56.4			19	1.94 F	1.49
												19	1.68	1.90
	4	100+§	31.5	8	6	61	F	27.2	60.6	17.3 ±4.13	<0.01	21	1.50†	2.05
	5	101	29.4	5	5	62	M	24.2	46.4	22.4 ±9.34	0.05			
	6	43	30.0	4	10	62	F	28.6	60.6	15.9 ±3.45	<0.01			
	7	100+§	89.1	5	6	63	F	22.8	71.0	24.1 ±3.40	<0.01	19	1.37†	1.64
	8	51	31.2	4	19	64	F	32.3	52.0	10.9 ±4.76	0.03			
	9	52	31.0	7	10	78	F	32.0	70.0	19.2 ±4.54	<0.01			
	10	51	33.1	7	5	123	MF	39.0	66.9	15.6 ±4.76	<0.01			
1B	11	23×6	92.0	3	5	33	F	9.3	49.2	20.7 ±2.93	<0.01			
	12	17×7	43.0	8	5	62	M	18.3	33.3	7.6 ±2.78	0.02	22	1.78‡	2.92

* 100 males were used in series 5 and 50 of the designated sex in all others.
† Worms were 18 days old.
‡ Worms were 21 days old.
§ The number of cercariae varied between 100–300 per mouse. Mice were exposed percutaneously in small jars.

even more stunted and were frequently extremely small (fig. 2).

Experiment 1B (series 11–12): initial repeated exposure to male and female cercariae and one test superinfection to male or female cercariae.—Two series of

mice were exposed initially to 23 and 17 cercariae (mixed sexes) weekly for 6 and 7 weeks, respectively. They were tested 7 and 28 days after the last exposure or 33 and 62 days after the first exposure with 50 female and 50 male cercariae, respectively. In these series, 9 and 18% of the worms were recovered as compared to 49 and 33% in the controls (fig. 3). The differences between corresponding immune and control groups gave a value of $P<0.01$ and $=0.02$ (table 1). In addition, growth of both male and female worms was retarded in the test mice.

Experiment 2 (series 13–20): one initial exposure to female worms and one challenging exposure to male or in one series, to male and female or female cercariae, respectively.—The female of *S. douthitti* will develop to sexual maturity and deposit parthogenetic eggs in the tissues of the host in the absence of male schistosomes (Short, 1947). This behavior was of value in the following series. Mice infected once initially with 34 to 100+ female worms were reexposed 9 to 117 days later to male worms (table 2). In series 18 and 19 females and male and female worms were used. Resistance to reinfection was evidenced at 61, 63 and 117 days as shown in significant differences in recovery of worms (table 2, fig. 4), but the worms

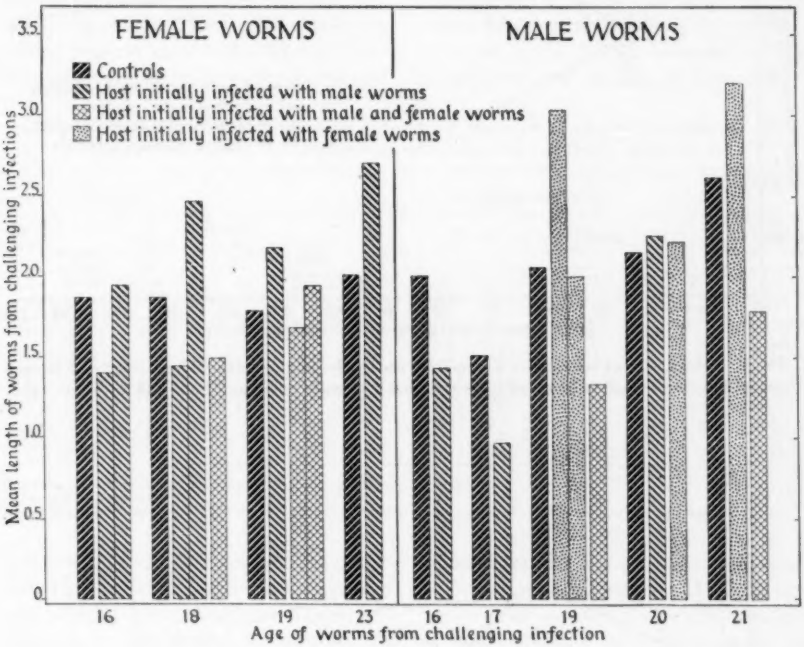

FIG. 2.—The average length of male and female worms developing from the challenging infections in experimental and control mice autopsied after 18 to 21 days. Each bar for the control represents worms of the same age recovered from several series. Each bar of the experimental mice represents worms of the same age recovered from individual series.

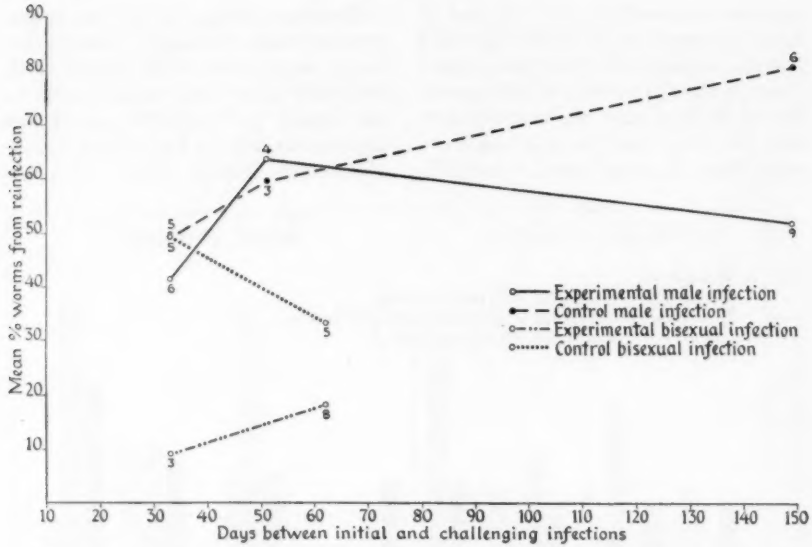

FIG. 3.—Mean percent recovery of worms from the challenging infection in mice infected 5 or more times initially with small numbers of male and female or male cercariae 33 and 149 days previously.

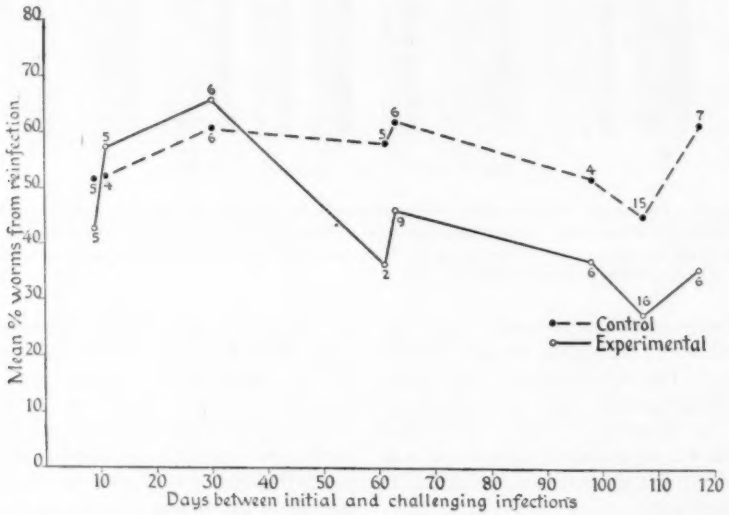

FIG. 4.—Mean percent recovery of worms from the challenging infection in mice infected with female worms 12 to 117 days previously.

of both sexes developed normally and grew as large or larger than the controls (table 2, fig. 2). At 98 days, the difference between the immune and control groups was not statistically significant ($P=0.08$). This finding may have been due to the wide range in number of worms recovered in the 4 controls, i.e., 26, 31, 33 and 16. The results of the preceding experiments with different combinations of sexes used for the initial and 14.1 ± 1.74 worms from 11 immunized mice not given the test challenging dose. Analysis showed that the difference of 8.89 worms between the number recovered in the immunized and challenged mice and the sum of both control groups is highly significant ($P \leqq 0.01$). Measurements of the lengths of worms recovered from the challenging infection were not made, but at necropsy of the immunized and challenged mice, unde-

TABLE 2.—*Experiment 2, consisting of 8 series of mice showing acquired immunity to superinfection 61 to 117 days after an initial infection with female cercariae.*

Exp. no.	Series	Initial exposure to one sex: Number of cercariae	Initial exposure to one sex: Mean worms recovered at autopsy	Challenging exposure*: Number of mice autopsied: Infected	Number of mice autopsied: Normal	Days after first exposure	Sex of worms	Percent worms recovered at autopsy: Infected	Percent worms recovered at autopsy: Normal	Percent worms recovered at autopsy: Mean difference	Percent worms recovered at autopsy: P	Mean length of worms in mm: Age of worms	Mean length of worms in mm: Infected	Mean length of worms in mm: Normal
2	13	100	66	4	5	9	M	42.7	51.6	8.85 ± 8.62	0.3			
	14	100+‡	144	5	5	11	M	57.2	52.2			16	2.54†	2.03
	15	35.5	19	6	6	30	M	65.9	61.8			21	3.21	2.48
	16	100+‡	15	2	5	61	M	36.6	58.6	8.12 ± 2.72	<0.01	19	3.04	2.29
	17	34	21	9	6	63	M	46.2	62.7					
	18	37	19	6	4	98	MF	37.0	51.7	7.17 ± 3.48	0.08	19	2.01	2.09
	19	45	14	16	15	107	F	27.4	45.2	8.9 ± 3.23	0.01			
	20	34	17	6	7	117	M	35.7	61.4	13.1 ± 4.44	0.01	20	2.22	1.75

* 100 females were used in series 13 and 50 of the designated sex in all others.
† Worms were 15 days old.
‡ The number of cercariae varied between 100–300 per mouse. Mice were exposed percutaneously in small jars.

test infections made it of interest to use female worms for both immunization and pretesting immunity. It is, however, difficult to distinguish females of the original and challenging infection given 16 to 25 days later. This difficulty was in part overcome by counting all the worms in challenged mice and comparing this number to the average total number in nonchallenged immunized mice and controls. Thirty mice were exposed to 45 female cercariae, and 107 days later, 16 of these and 16 normal mice were challenged with 50 female cercariae. At necropsy 16 days after the challenging exposure 27.87 ± 2.04 worms were recovered from 16 immunized and challenged mice as compared to 22.6 ± 1.95 worms from 15 control mice and veloped female worms were recovered.

Experiment 3A (series 21–29): exposure once initially to male worms and at superinfection to female, male, or male and female worms.—A large number of mice were initially infected percutaneously in small containers with 100 to 300 cercariae per animal. Many of these mice receiving the largest number of cercariae died within 60 days. A second group of mice were exposed to 100 counted cercariae placed on the clipped abdomen. These mice averaged 61 worms on the 35th and 188th day after infection as ascertained by an autopsy of 15 mice, and gained weight at the same rate as did the controls. Groups of 4 to 8 mice together with groups of normal mice were then challenged 15 to 211

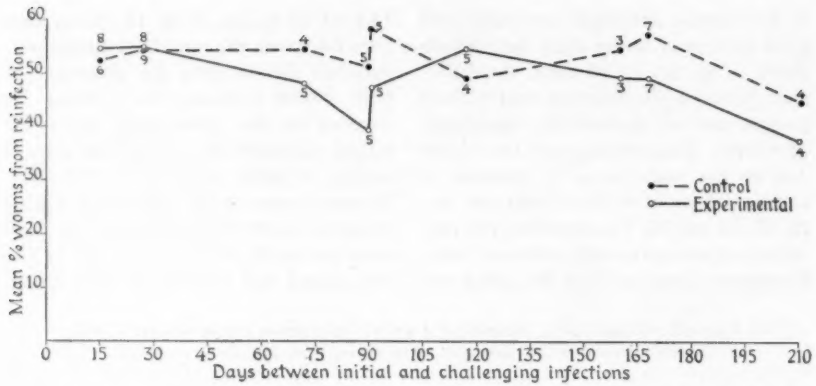

FIG. 5.—Mean percent recovery of worms from the challenging infection in mice infected with male worms 15 to 211 days previously.

days later with 50 female, male, or male and female cercariae. At autopsy, from 37% (series 29) to 55% (series 22) of the worms of the initial infection were recovered in the infected groups (fig. 5). In spite of the persistence of worms from the original infection in infected mice no significantly fewer worms were recovered in them than in the control groups (fig. 5, table 3). Measurements of the recovered worms, however, indicated that female worms develop normally whereas males are stunted (fig. 2).

Experiment 3B (series 30–32): initial repeated exposure to male cercariae and one test superinfection with cercariae (variously combined as to sex).—Three series of mice were immunized as follows: Series 30 was exposed to an average of 23 cercariae 3 times at 4-day in-

TABLE 3.—*Experiment 3, consisting of 12 series of mice exposed to initial infections with male schistosomes.*

Exp. no.	Series	Initial exposure to one sex		Challenging exposure*										
		Number of cercariae	Mean worms recovered at autopsy	Number of mice autopsied		Days after first exposure	Sex of worms	Percent worms recovered at autopsy				Mean length of worms in mm		
				Infected	Normal			Infected	Normal	Mean difference	P	Age of worms	Infected	Normal
3A	21	100	69	8	6	15	F	54.3	52.2			25	2.77	2.06†
	22	100+*	92	8	9	27	F	54.8	54.5			22	2.18†	1.99
	23	50	17	5	4	72	F	48.2	54.4	2.7±8.33	>0.5	17	2.22‡	1.94
	24			5	5	90	M	39.4	51.3	6.2±2.75	0.06	17	0.97	1.52
	25	100+*	168	5	5	91	MF	47.6	58.3	5.0±5.55	0.4	16 F 16 M	1.41 1.43	1.86 1.98
	26	100+*	88	5	4	117	F	54.5	49.2			23	2.7	2.02
	27	100+*	107	3	3	160	MF	49.3	54.6	2.7±2.74	0.5	16	1.94	1.86‡
	28	100	53	7	7	168	MF	49.4	57.2	3.8±3.10	0.2	20 F 20 M	2.14 2.25	2.59 3.15
	29	100	59	4	4	211	MF	37.5	44.8	3.5±2.99	0.3			
3B	30	23×6	75	6	5	33	F	41.3	49.9	4.2±3.85	0.3	18	1.45	1.47
	31	60×3	148	4	3	51	F	63.8	59.9					
	32	22×7	57	9	6	149	F	52.2	81.4	14.1±3.85	<0.01	18	2.47	2.03

* The number of cercariae varied between 100–300 per mouse. Mice were exposed in small jars.
† Worms were 20 days old.
‡ Worms were 19 days old.

tervals and 3 times at weekly intervals. They were challenged 7 days after the last of the initial series of exposures or 33 days after the first exposure with 50 female cercariae. Six normal control mice were infected similarily at this time. Series 31 involved 4 immune and 3 control mice of the *dba* strain. As far as the data go, this strain behaved in general as did the Carworth Farms strain. The initial infection consisted of exposing a group of 10 mice to 500 to 1000 male cercariae in a battery jar each week for 6 weeks. This treatment killed 6 mice half way through the immunization. The challenging superinfection of 50 females was given 18 days after the last exposure and 51 days after the first exposure. Four controls were infected at this time. In series 32, 23 mice were exposed to 20 to 25 male cercariae once a week for 7 weeks. Then, 107 days after the last exposure and 149 days after the initial exposure, 9 mice were subjected to 50 female cercariae.

The results of this experiment were essentially similar to the results in experiment 3A. Forty-one, 63 and 52% of worms from the challenging infection were recovered in the infected mice and 49, 59 and 81% in the control groups (fig. 3). The difference between 52% and 81% in series 32 gave a probability of 0.01 (table 3), but the conclusion seemed unwarranted that an immunity was acquired in the infected group because 81% was unusually high for the control group and 52% in the infected group fell within the expected range.

DISCUSSION

The foregoing experiments demonstrate that mice initially infected with male and female *S. douthitti* develop a partial immunity to superinfection after 30 to 60 days and that the immunity lasts as long as 120 days (end of test period). The partial immunity was shown by a statistically significant reduction in number of worms and a stunting of worms in the challenging infection as compared to control mice. A lesser degree of immunity resulted in mice initially infected with either female or male worms as compared to mice infected initially with mixed males and females. Mice immunized by infecting with females showed a reduction in worms of the challenging infection, but no stunting of males. Mice infected with males showed no reduction in number 15 to 211 days after initial infection but a stunting of males and not females. These results are not in accord with the results published by either Vogel (1949) or El-Gindy (1950). Vogel reported that, of two monkeys infected with male worms of *S. japonicum*, one developed a strong and the other a partial resistance to superinfection in a year and a half. He therefore concluded the egg is not the antigenic stimulus in schistosome immunity. In the present experiments, the last test of immunity in male worm-infected mice was at 211 days, but it seems probable to the author that immunity should have been demonstrated by this time in view of the fact that it occurred after 30 days in mice infected with a mixture of sexes. El-Gindy (1950) reported a more selective type of resistance against female worms. Evans and Stirewalt (1951) demonstrated for experimental infections with *S. mansoni* that the infectivity of the cercariae shed from one snail varied with the age of the snail and the physiological balance between parasite and host. This finding would indicate that each experiment should be separately controlled and that mice exposed to cercariae from different snails could only be tentatively compared. The time at which the percent recovery of worms of the test infection is ascertained is important. Although some authors believe that the route of migra-

tion from the lungs to the portal system is vascular others think that it may be in part somatic. I have found that autopsies of infected mice performed before 15 to 16 days do not yield all the worms. Data from El-Gindy's control series corroborate this conclusion. From mice autopsied 2 to 13 days after infection, only 18 to 45% schistosomulae were recovered, whereas 63 to 89% were recovered 14 to 30 days after exposure. Since some schistosomulae are not recovered early in infection by perfusion they may migrate somatically. El-Gindy's results are not in accord with mine perhaps because he did not control each experiment individually and autopsied a majority of his mice 13 days or earlier.

The lapse of about a month between infection and the development of immunity in mice coincides with the production of many eggs by female worms. The lack of immunity in male infections supports the hypothesis that the production of eggs by the female worm may well be the antigenic stimulus in the mouse. Antibodies in immune serum from helminth infections act largely as precipitins in studies made on *Nippostrongylus*, *Ancylostoma*, *Strongyloides* and *Trichinella* (Taliaferro, 1943). Membranes forming around schistosome cercariae in immune serum (Vogel and Minning, 1949a and b; Papirmeister and Bang, 1948) may also be formed by the action of precipitins in the serum. The mechanism of immunity in nematode, cestode and trematode parasites are probably very similar. That the egg is the antigenic stimulus in the development of precipitins associated with antilarval activity in *Ascaris lumbricoides* infections in rabbits was shown by Oliver-González (1943). According to him, antibodies in immune serum from rabbits fed with ascaris eggs were absorbed by whole worm antigen. Among serums prepared against isolated tissues of the adult worm only the anti-egg serum absorbed the antibody. Moreover, larvae placed in anti-egg serum are immobilized and in no other anti-tissue serum.

In many of the mice infected with a single sex, challenging infections of the opposite sex were made so that the worms of the first and second exposures could be separated without error. In the series injected with male and female worms and in those experiments where worms of the same sex were used in the challenging exposure, worms were usually separated on the basis of size and sexual development. The percent recovery of worms from primary infections compared to recovery from challenging exposures in controls was similar. Thus, in 16 experiments where the cercariae were counted in the primary infections, an average of 53.3% cercariae were recovered in 92 mice which were later superinfected and 57.6% in control mice infected when the immune mice were superinfected. The percent survival of infected mice compared to survival of control mice was similar. Thus in 31 experiments 211 infected mice were used and 82.46% were autopsied, and of 213 control mice 86.85% were autopsied.

Development of male and female schistosomes in immunized and nonimmune hosts with various sexes of worms is shown in figure 2. A total of 632 female worms and 456 male worms from 37 mice recovered from control animals autopsied the same day after superinfection were measured and used as the standard. In addition, 442 female worms and 72 male worms from 20 and 8 mice, respectively, in the male infected series, 45 female and 53 male worms from 5 and 8 mice respectively, in the bisexual series and 190 male from 10 mice in the female series were measured. Worms re-

covered from individual series of the same age group were graphed separately. They often show a disparity in size because they are elastic and extend and contract easily. The variability is shown, for example, by the 2 groups of female worms at 18 days. In spite of this variability, the following definite trends are evident. Female worms in male infections and male worms in female infections are slightly larger than the controls. This finding indicates normal development since the population of males or females when grown separately are smaller than when grown together. Male and female worms developing in immune animals infected initially with both sexes and male worms developing in mice infected initially with male worms were stunted in growth (cf. worms from immune mice with controls in figure 2).

SUMMARY

Immunity to superinfection was acquired by mice (CFI, Carworth Farms strain) against *Schistosomatium douthitti* 35 to 127 days (end of test period) after an initial infection as ascertained by the recovery of schistosomes 16 to 25 days after a challenging superinfection of 50 cercariae (a mixture of both sexes). Resistance in mice is increased by a repeated immunization with a series of exposures at 5- or 6-day intervals for several weeks. Both male and female worms developing from the challenging infection are reduced in number and stunted in growth as compared to worms in control mice.

An initial infection of female worms produced an acquired immunity to superinfection when tested after 60 to 121 days (end of test period) by a superinfection with male, female, or male and female worms as indicated by their reduced number but not by a retardation of their development.

An initial infection of male worms in all but one experiment did not induce resistance to superinfection, as indicated by no reduction in number in the challenging infection. Male worms in the challenging infection, however, were stunted but females developed normally.

Quantitative techniques were used in challenging mice and all experiments were individually controlled.

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SEROLOGICAL DIAGNOSIS OF INFLUENZA: ANTIBODY LEVELS IN RELATION TO THE CONCENTRATION OF VIRUS USED IN THE HEMAGGLUTINATION-INHIBITION TEST

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In order to clarify certain significant factors in the serological diagnosis of influenza by the modified method of Hirst and Pickels,[2] the present study was undertaken. Over a period of seven months changes in the titer of a control serum were observed from day to day, using three strains of influenza antigens. Therefore, this investigation was conducted to determine if these variations in the influenzal antibody titer of pooled human serum were influenced primarily by the virus concentrations used in the hemagglutination-inhibition test. It was also considered essential to demonstrate the effect different virus concentrations had upon the antibody rise between acute and convalescent serums.

The data presented substantiate the findings of others[1–4] by showing that the level of influenzal antibody varies inversely with virus concentrations. In addition, varying the virus concentration in a controlled experiment has been found not to affect the antibody rise between acute and convalescent serums.

MATERIALS AND METHODS

Antigens.—PR-8, Lee, and FM-1 influenza viruses were used as representative strains of types A, B, and A′, respectively. During the first half of this study these antigens were procured from Lederle Laboratories in the partially purified and concentrated form. Thereafter these strains were prepared in this laboratory by inoculating the allantoic cavity of 11-day-old chick embryos with 0.1 ml of a suitably diluted virus. Following 48 hours of incubation at 35 C the eggs were placed at 4 C overnight. The allantoic fluid was harvested aseptically and stored at 3 to 4 C in bottles with ground glass stoppers until needed. The agglutinating activity of each lot was determined in terms of Hirst's units and the allantoic fluid diluted to contain a concentration of 16 to 24 units/ml. Following dilution in saline, additional titrations of the antigens were conducted previous to running the serums. Frequently it was necessary to again adjust the virus concentration by addition of either infected allantoic fluid or saline.

Standard serum.—Serum for the standard control was obtained by drawing 15 ml of blood aseptically from each of 100 naval recruits in the spring of 1949. Following separation of the serums, 5 ml aliquots were pooled and mixed.

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We wish to express our appreciation to the Department of Biometrics for their aid and advice throughout the course of this work.

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From the pool thus formed, 10 ml amounts were stored at −8 C in sealed ampules. Vials of serum were maintained in the frozen state with exception of the one in daily use. This sample was stored in a stoppered tube at 3 to 4 C and served as a control in daily titrations.

Chicken red cell suspension.—Chicken blood was obtained aseptically from young, adult, white Leghorn roosters by cardiac puncture. From 15 to 25 ml were drawn from each of 3 to 5 roosters and mixed with an equal volume of Alsever's solution. The resultant mixture was stored at 3 to 4 C until used. Cells less than 24 hours or more than 7 days old were not used in the test. The 1.5% red cell suspensions were prepared daily and were not used the second day. Following the first centrifugation, the red cells were washed three times with physiological saline and packed on the last washing. Centrifugation was at 1300 r.p.m. for 8 minutes in all instances. Volumes of red cells required for dilution were made by reading the graduations on the 15-ml centrifuge tubes. Red cell counts of the 1.5% suspensions were occasionally made. These were found to vary from 95,000 to 100,000 rbc/cmm.

Prior to determining the antibody titer on any serum, the Coleman Junior Spectrophotometer Model 6A was adjusted to red cell control tubes. These tubes contained 3 ml of normal saline to which was added 1 ml of the 1.5% chick cell suspension. The concentration of cells in the tubes was then 0.37% or 50% of the cells present in the test. These control tubes were mixed by inverting twice and allowed to stand for 75 minutes. Because the optical density of the 0.75% test cells after standing for 75 minutes is equal to 100%, the spectrophotometer was adjusted to a reading of 50% light transmission by use of the control tubes.

Method of titration.—Influenza antibody levels of all serums and the standard serum controls were determined, using the method described by Hirst and Pickels.[2] All serums were diluted 1:8 and inactivated at 56 C for 30 minutes. Serial transfers of the serum into saline were performed by delivering 0.5 ml amounts with calibrated Cornwall pipettes, making the final dilution in the first tube 1:32 after the addition of 0.5 ml of antigen and 1.0 ml of chicken red cells. The 50% inhibition end points were read spectrophotometrically at 542 mμ. During the incubation period of 75 minutes the temperature in the laboratory was maintained between 23 C and 27 C.

RESULTS

The relationship between the chick cell agglutination (CCA) titers of the viruses, expressed in Hirst's units, and the corresponding antibody titers of the serum pool are shown in figures 1 and 2. Each point on these diagrams represents an observation of which no two were made on the same day. For purposes of brevity and because of similar antigenic patterns, types A and A′ viruses are incorporated together in figure 1. When the ordinate and abscissa are scaled logarithmically in figure 1, it is seen that the result is approximately a straight line. The titer values greater than 1700 are those which fall furthest from the line of regression. From the positions of these points it would appear that they fitted about another straight line more distant from the abscissa. These same data produce curvilinear relationship if plotted on an arithmetic scale.

The inverse relationship between the concentrations of type B influenza virus and the corresponding serum titers obtained presents a similar picture in figure 2. The straight line of figure 2 lies somewhat closer to the ordinate than does the line for figure 1, and is due to the low titer of the serum to type B virus.

In the summary of these data obtained from the 195 observations (table 1) it is shown that the correlation coefficients between the concentrations of viruses used and the antibody titers of the serum are in each case statistically significant. The mean values for unitage of the viruses exceed the maximum desired concentration of 24 units by less than a one-half tube difference. This would imply that during the time these determinations were made a tendency to use an excess of antigen existed in the laboratory. Table 1 further shows that the serum pooled from the initial blood of 100 naval recruits in the spring of 1949 was type A′ specific.

To determine if these results would

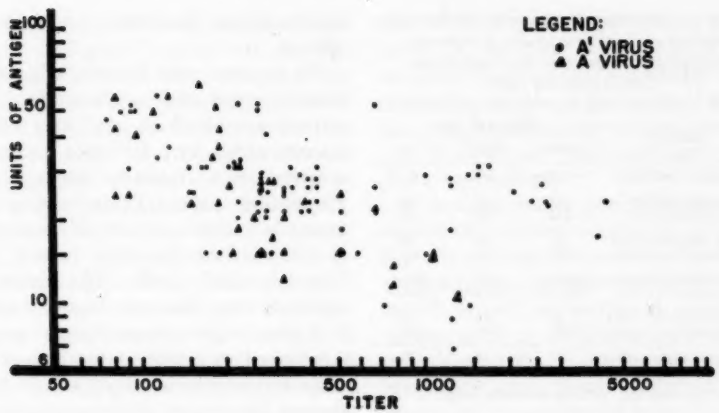

FIG. 1.—Comparison of influenza types A and A′ virus concentrations to homologous antibody titers obtained by the method of Hirst and Pickels.

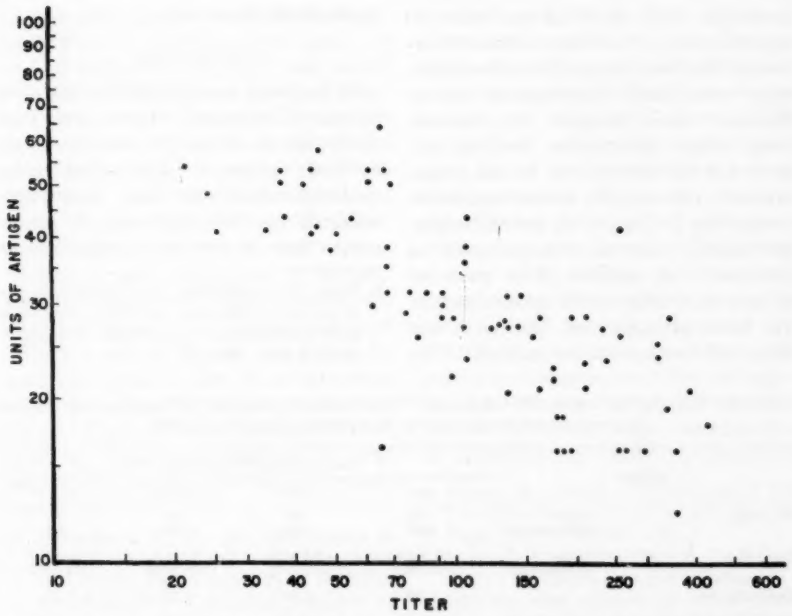

FIG. 2.—Comparison of influenza type B virus concentration to the homologous antibody titer obtained by the method of Hirst and Pickels.

TABLE 1.—*Summary of data when the hemagglutination units of three strains of influenza viruses are compared to their homologous serum antibody titers.*

Statistic	Strains of virus		
	PR-8	FM-1	Lee
Correlation coefficient	−0.60	−0.44	−0.60
Mean units/ml virus	32	32	34
Mean antibody titer	235	355	89
Median units/ml virus	32	33	35
Median antibody titer	220	317	117
Range units/ml virus	12–64	7–58	10–64
Range antibody titer	80–1350	48–2700	22–418
S.D.M.* units/ml virus	20	13	14
S.D.M. antibody titer	228	279	103
Number of observations	37	81	77

* S.D.M. = standard deviation about the mean.

hold true experimentally a protocol was designed to show the influence of varying PR-8 virus concentrations upon the homologous antibody titers of two pools of human serum. The pools were formed to simulate acute and convalescent specimens, and an effect was made to approximate a two-tube difference between the two pools. Fifty-nine separate "acute" and "convalescent" serum dilutions were titrated in alternate rows, using the routine method employed in this laboratory. In the preparation of the virus dilutions, concentrations of 10, 20, 30, 40, 50, and 60 hemagglutinating units/ml were prepared as accurately as possible. Five pairs of serums were then tested against each of the virus preparations. One pair was discarded because of an accident. The results of the experiment are shown in table 2.

In practice the desired concentrations of virus were approximated but not always obtained, and as a consequence these data in table 2 are assembled in 4 arbitrary ranges of agglutinating units. There was a decrease in antibody titer with an increase in virus unitage for both "acute" and "convalescent" pools. The range in antibody titer that was encountered in 6 of these 8 titer ranges shown in table 2 is from 1 to 2 tubes, regardless of the virus concentration employed. In 2 instances the ranges slightly exceeded 2 tubes. A most striking result is the evidence that the quantity of virus used had no effect upon the degree of rise in titer between the pools. This is shown by the close agreement in both the mean tube rises and the standard deviations about the means.

DISCUSSION

It has been recognized that the combining of influenza viruses with their antibodies is similar to other antigen-antibody reactions. The classical finding of Dean and Webb[5] that "in antigen-antibody reactions the point of optimal proportions is the same regardless of

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TABLE 2.—*The effect of various PR-8 influenza virus concentrations on the antibody rise between acute and convalescent serum pools by the method of Hirst and Pickels.*

Statistic		Units per ml of PR-8 influenza virus			
		8–15	16–31	32–63	64–128
Mean titer	Acute	724	531	184	161
	Convalescent	2416	1386	575	465
Range titer	Acute	512–1024	182–1024	91–338	64–275
	Convalescent	1175–4096	676–2200	338–1024	208–1024
Mean tube rise		1.56	1.60	1.63	1.52
Standard deviation about the mean tube rise		±0.57	±0.45	±0.57	±0.60
Number of observations		7	7	26	19

the volume in which the reaction takes place" has been verified for red cell agglutination-inhibition reactions by Hirst.[1] It has also been demonstrated by Hirst[1] that a near inverse proportion exists between the virus concentration used and the influenza antibody titer in red cell agglutination-inhibition tests. Whitman[3] confirmed this observation and showed conclusively that in hemagglutination-inhibition tests, constant-virus serum titrations or constant-serum virus titrations can be used to determine antibody levels interchangeably, providing red cell concentration and volume of reagents remains constant. Horsfall[4] has also shown that in the mouse neutralization test a linear relationship likewise exists. The first two figures above substantiate these observations by showing an inverse relationship between the antigen concentration used in the test and the antibody titer of a human serum pool.

Stuart-Harris[6] observed that blood cells from different fowl produce different titers to influenza type B virus but not to type A. Also, the seasons of the year introduced an affect upon the cells. Salk[7] and Hirst[1] have reported a shift in the chick cell agglutination-inhibition end point when the concentration of test cells varied. Whitman,[3] using human type O cells, demonstrated that variations in cell concentration of 2%, 1% and 0.5% did not affect the antibody titer of immune ferret serum when low virus dilutions were used, but as the dilution factor increased divergent end points were observed. A large source of error is nevertheless present when different lots of red cells are introduced in the test.[3] Even though our spectrophotometer was adjusted to each particular lot of red cells, we must admit that this method gives no precise control between any two separately prepared suspensions.

It is of interest to note that though a significant correlation existed between the virus concentrations used and the antibody titers obtained (table 1), the results at any antigen level ranged in values up to a difference of about 2 tubes (table 2). A 2-tube variation in range has also beeen repeatedly observed by Stuart-Harris[6] when he used the same concentration of viral agglutinating units.

As stated previously, a number of the titers of the serum pool to type A′ virus are remote from the line of regression (fig. 1). A perusal of laboratory work sheets revealed that these titers occurred on consecutive days. Furthermore, their appearance coincided with the development of a heavy precipitate in the type A′ infected allantoic fluid. It has been repeatedly observed that when heavy precipitates develop in infected allantoic fluid, the inhibition titer of serum to the agglutinating forces of the viruses is altered.[8,9] The location of these points in figure 2 shows that in these instances the antibody titer of the serum ranged from approximately 1000 to 4400. In comparison, the values of the points that approximate the line of regression ranged from approximately 200 to 600 for the same concentrations of agglutinating units. Normally the

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CCA titer of infected allantoic fluid is reduced as precipitates develop. This, in turn, requires that a greater concentration of the allantoic fluid be used to obtain the desired viral unitage which increases the amount of nonviral protein in the test. Since work recently completed in this laboratory has shown that nonviral protein interferes with red cell-virus combinations, one might postulate that the cause for these high inhibition titers was the antigen.[9]

The serological results from the experiment on "acute" and "convalescent" pools are interesting and significant. These results demonstrate that the virus unitage controls the level of the antibody titer. The degree of antibody rise, however, between acute and convalescent serums remains unchanged. The close similarities in the mean tube rises in this experiment enhance the value of the test when used for diagnostic purposes.

The results presented here indicate that when influenzal antibody titers of any serums determined by this method are compared from day to day and between laboratories it would be helpful to know the virus unitage employed in the test.

SUMMARY AND CONCLUSIONS

1. Data from 195 influenzal antibody titrations determined by the method of Hirst and Pickels upon a pool of human serums is presented.

2. The relationship between the concentrations of types A, B, and A′ influenza viruses to the homologous antibody titers of the serum pool was inverse.

3. Results of an experiment are presented showing that though virus unitage influences the level of antibody, the degree of rise between acute and convalescent serum pools remains essentially unchanged.

4. In comparing influenzal antibody levels from day to day or between laboratories, the concentration of the virus used in performing the test should be considered.

INTERFERENCE BETWEEN ST. LOUIS ENCEPHALITIS VIRUS AND WESTERN EQUINE ENCEPHALOMYELITIS VIRUS ALONG A NEURONAL PATHWAY

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It has been observed on a number of occasions that the presence of one virus within an organism may prevent or inhibit the multiplication of a second virus in that same organism. This so-called "interference" between one virus and another has been termed the "interference phenomenon." The first observations on "interference" between animal viruses were reported in 1935. In that year Hoskins[1] reported that a neurotropic strain of yellow fever virus would protect monkeys against a subsequently inoculated viscerotropic strain of yellow fever virus, and Magrassi[2] reported that a non-encephalitogenic strain of herpes virus would protect rabbits against an encephalitogenic strain subsequently inoculated. Since 1935 a number of papers have appeared in which "interference" between various viruses has been demonstrated. Extensive reviews of the literature on the "interference phenomenon" have already appeared[3–8] and no attempt will be made at this time to review the literature on this subject.

The studies reported here were undertaken to determine whether the passage of one neurotropic virus over a nerve pathway would prevent or interfere with the passage of a second neurotropic virus over the same nerve pathway. St. Louis encephalitis (S.L.E.) and Western equine encephalomyelitis (W.E.E.) viruses were selected for the study because it had already been demonstrated that S.L.E. virus could interfere with the multiplication of W.E.E. virus.[9] Furthermore, it was important for such a study to use one virus which, although it could invade the central nervous system (C.N.S.) along a given nerve pathway, would not initiate a fatal infection in the animal being used. In addition, it was desirable to employ a second virus which would invade the C.N.S. by way of the same nerve pathway as the first but which would initiate a fatal

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infection upon reaching the C.N.S. It has been demonstrated that S.L.E. virus when instilled into the noses of 21-day-old rats invades the C.N.S. by way of the olfactory tract. Despite the fact, however, that the virus invades, multiplies and becomes disseminated throughout the C.N.S. of rats of this age, there is never any clinical evidence of infection.[10,11] On the other hand, W.E.E. virus, following nasal instillation, rapidly invades the C.N.S. of 21-day-old rats by the olfactory route and causes a fatal encephalitis in animals of this age.[12] Becuase of the behavior of S.L.E. and W.E.E. viruses in the rat following intranasal instillation of the viruses and also since it had already been established that S.L.E. virus could "interfere" with W.E.E. virus, these viruses were selected for study, using the rat as the experimental animal.

MATERIALS AND METHODS

Viruses and animals.—The Webster no. 3 strain of S.L.E. virus used in the studies was obtained from Dr. Albert B. Sabin. The W.E.E. virus was obtained from the same source. Both of these viruses had undergone an unknown number of intracerebral passages in mice. The virus suspensions used in the present study were prepared from virus-infected brains of 3- to 4-week-old Rockland Farms Swiss mice. The infected brains were ground in a mortar with sand and sufficient inactivated, undiluted rabbit serum to make a 10% suspension of the infected brains. The suspensions were centrifuged at about 2000 r.p.m. for 5 minutes to sediment gross particles. Immediately after centrifugation the supernatant fluid was removed and distributed in 2 ml amounts in screw-capped vials. The virus in the vials was "quick-frozen" by rolling the vials in a mixture of dry-ice and alcohol. The vials containing the frozen virus were then stored in a dry-ice cabinet. As virus was needed one or more vials were removed from the dry-ice cabinet and thawed in a water bath at 37 C just before it was to be used. The rats used in the experiments were 3- to 4-week-old male Sprague-Dawley.

Virus titrations.—The various virus suspensions were titrated intracerebrally in 3- to 4-week-old Rockland Farms Swiss mice, using a mixture of 10% rabbit serum in saline as a diluent. Two preparations of each of the viruses were used. The LD_{50} titers[13] of the two suspensions of S.L.E. virus were $1\times10^{-8.3}$ and $1\times10^{-7.8}$, whereas the titers of the two W.E.E. virus suspensions were $1\times10^{-9.3}$ and $1\times10^{-8.2}$.

EXPERIMENTAL

Interference between S.L.E. and W.E.E. following nasal instillation of both viruses.—Duffy and Sabin[10,11] found that S.L.E. virus could be found in the anterior rhinencephalon of 21-day-old rats as early as 24 hours following nasal instillation of the virus but that the virus did not reach its highest concentration in this part of the brain until 3 days after the virus had been instilled into the nose. They also found that the virus could be detected in the "remainder of the brain" as early as 3 days following nasal instillation.

Since the rats used in the experiments reported here were of a different strain from those used by the above authors, a preliminary experiment was conducted to follow the progression of nasally instilled S.L.E. virus into the C.N.S. of 3- to 4-week-old rats, and to determine whether the 3- to 4-week-old rats being used would develop a clinical infection following inoculation of the virus by the nasal route. Eighteen 3- to 4-week-old rats were inoculated intranasally with 0.05 ml of a 10% suspension of S.L.E. virus. Two rats were sacrificed on each of the days indicated in table 1. The brains of the animals were removed and a 10% saline suspension of the pooled anterior rhinencepha-

10. Duffy, C. E. and Sabin, A. B. 1942, Behavior of St. Louis encephalitis virus in rats. J. Bact. **43**: 88 (Abstract).
11. Duffy, C. E. and Sabin, A. B. Unpublished data.
12. Duffy, C. E. Unpublished data.
13. Reed, L. J. and Muench, H. 1938, A simple method of estimating fifty percent end points. Am. J. Hyg. **27**: 493–497.

lons and the pooled "remainders of the brain" inoculated intracerebrally into 3- to 4-week-old mice to determine whether virus was present. The data presented in table 1 show that S.L.E. virus was present in the anterior rhinencephalon as early as 24 hours following inoculation of the virus and that it was still present on the 6th day, when the experiment was terminated. Virus was not found in the "remainder of the brain" until the 4th day following inoculation of the virus. The remaining 8 rats were observed for 30 days and never showed any evidence of infection.

Experiments were now conducted to determine whether S.L.E. virus, having passed over the olfactory tract and being present in the brain, would interfere with the passage of W.E.E. virus over the same pathway. Thirty-two 3- to 4-week-old rats were used. Sixteen of these animals were inoculated intranasally with 0.05 ml of a 10% suspension of S.L.E. virus, and the other 16 received 0.05 ml of a 10% suspension of normal mouse brain by the same route. Seventy-two hours later all 32 rats were inoculated intranasally with 0.05 ml of a 10% suspension of W.E.E. virus. The results appear in tables 2 and 3. It will be seen (table 2) that all of the 16 rats which received the normal mouse brain suspension and then W.E.E. virus 72 hours later developed a fatal infection. On the other hand, in the group of animals which received S.L.E. virus, followed 72 hours later with W.E.E., only 8, or 50%, of the rats succumbed. In comparing these two groups of animals it will be noted that 7 of the animals which received normal mouse brain followed by W.E.E. virus were dead on the 3rd day and the remaining animals died on the 4th day. In the group of rats which received both S.L.E. and W.E.E. viruses the course of events was different, since of the 8 rats which succumbed, none of the animals died

TABLE 1.—*Progression of S. L. E. virus from nose to C. N. S. of rats.*

Day sacrificed after injection of S.L.E. virus	Rat no.	Parts tested for presence of virus	
		Anterior Rhinencephalon*	Remainder of brain*
1	1 and 2	+	−
2	3 and 4	+	−
3	5 and 6	+	−
4	7 and 8	+	+
6	9 and 10	+	+

+ Indicates presence of virus as determined by inoculating material intracerebrally into mice.
− Indicates absence of virus as determined by inoculating material intracerebrally into mice.
* Material from two rats pooled.

TABLE 2.—*Interference between S.L.E. and W.E.E. following intranasal instillation of both viruses.*

Number of rats tested	Material instilled into nose	Interval (days) between W.E.E. and death												Percent mortality
		1	2	3	4	5	6	7	8	9	10	11	12	
16	N.M.B., W.E.E.	0*	0	7	9									100
16	S.L.E., W.E.E.	0	0	0	0	0	0	2	0	1	1	3	1	50

N.M.B.—Normal mouse brain.
* Number of rats which died on that day.

TABLE 3.—*Interference between S.L.E. and W.E.E. following intranasal instillation of both viruses.*

Number of rats tested	Material instilled into nose	Interval (days) between W.E.E. and signs											Total number showing signs
		1	2	3	4	5	6	7	8	9	10	11	
16	N.M.B., W.E.E.	1	10	5									16
16	S.L.E., W.E.E.	0*	0	0	0	1	1	0	2	2	1	1	8

N.M.B.—Normal mouse brain.
* Number of rats first showing signs on that day.

before the 7th day following inoculation of W.E.E. virus and one animal survived until the 12th day. The results presented in table 3 show that the incubation period was prolonged in the animals which were inoculated with both viruses. It will be seen that the incubation period in the animals which received the suspension of normal mouse brain followed by W.E.E. virus was as short as one day and that by the 3rd day all 16 animals showed signs characteristic of infection with W.E.E.

intracerebrally.—Having demonstrated that S.L.E. virus protected 50% of the animals against W.E.E. when both viruses were inoculated intranasally, an experiment was performed to determine whether nasally instilled S.L.E. virus would protect rats against W.E.E. if the latter virus were injected directly into the brain of the S.L.E.-treated rats. Twelve 3- to 4-week-old rats were inoculated intranasally with 0.05 ml of a 10% suspension of S.L.E. virus. At the intervals indicated in table 4

TABLE 4.—*Effect of previous intranasal instillation of S.L.E. on W.E.E. injected intracerebrally.*

Number of rats tested	Interval between intranasal inoculation of S.L.E. and intracerebral injection of W.E.E.	Results					
		Day of death					Percent mortality
		1	2	3	4	5	
4	2 days	0	4				100
2	(W.E.E. only—Controls)	0	2				100
4	4 days	0	3	1			100
2	(W.E.E. only—Controls)	0	2				100
4*	6 days	0	0	2	0	2	100
1	(W.E.E. only—Controls)	0	1				100

* Two of these animals developed paralysis of both posterior extremities two days following intracerebral injection of W.E.E. They died on the third day.

virus. On the other hand, none of the 8 rats which received both S.L.E. and W.E.E. viruses, and subsequently succumbed, developed clinical signs of infection before the 5th day following nasal instillation of W.E.E. virus, and in one instance the incubation period was as long as 11 days.

The data presented in tables 2 and 3 suggest that if S.L.E. virus is instilled into the noses of 3- to 4-week-old rats, it will protect some of the animals against W.E.E. virus inoculated 72 hours later by the same route. In addition, when rats which have had both S.L.E. and W.E.E. viruses instilled into their noses succumb to the W.E.E. virus, they survive for a longer period than animals which have not been previously inoculated with S.L.E. virus.

Effect of intranasal instillation of S.L.E. on W.E.E. subsequently injected

the above rats were inoculated intracerebrally with 0.05 ml of a 1×10^{-6} suspension of W.E.E. virus. Normal rats of the same age were used as controls. It will be seen that the previous nasal instillation of S.L.E. virus did not effect the course of infection initiated by the W.E.E. virus. None of the rats which had been injected intranasally with S.L.E. virus and subsequently inoculated intracerebrally with W.E.E. survived. They appeared to be just as susceptible to the W.E.E. virus as were the control animals.

From the data so far presented it would appear that if S.L.E. virus is instilled into the noses of 3- to 4-week-old rats and 72 hours later W.E.E. virus is inoculated by the same route, the latter virus either is prevented from reaching the brains of a number of the animals, or if it does reach the brain

is "interfered" with, in some manner, so that it is unable to initiate a clinically apparent infection. Since nasally instilled S.L.E. virus did not protect the rats from W.E.E. virus inoculated intracerebrally, it would appear that S.L.E. virus reaching the C.N.S. by the olfactory route prevented the subsequent passage of W.E.E. virus over the same pathway in about 50% of the animals.

The distribution of W.E.E. virus in rats which received S.L.E. and W.E.E. viruses intranasally.—Fifty percent of the 3- to 4-week-old rats which were inreached the C.N.S. of these animals.

In a preliminary experiment, twelve 3- to 4-week-old rats were inoculated intranasally with 0.05 ml of a 10% suspension of S.L.E. virus, and 72 hours later these 12 animals received 0.05 ml of a 10% suspension of W.E.E. virus by the same route. At each of the intervals indicated in table 5, two of the rats were sacrificed. Blood from the two animals was obtained by cardiac puncture and pooled. Heparin was used as an anticoagulant. The brains and spinal cords of the two rats were removed.

TABLE 5.—*Distribution of W.E.E. in rats which received S.L.E. and W.E.E. viruses intranasally.*

Interval following W.E.E. when rats were sacrificed (days)	Rat no.	Parts tested							
		Anterior rhinencephalon* tested for presence of virus in		Remainder of brain* tested for presence of virus in		Spinal cord* tested for for presence of virus in		Undiluted blood* tested for for presence of virus in	
		Rats	Mice	Rats	Mice	Rats	Mice	Rats	Mice
1	1 and 2	+	+	−	+	−	−	−	−
2	3 and 4	+	+	+	+	+	+	−	−
3	5 and 6	−	+	−	+	−	+	−	−
4	7 and 8	+	+	+	+	+	+	−	−
5	9 and 10	−	+	−	+	−	+	−	−
6	11 and 12	+	+	+	+	+	+	−	−

+ Indicates presence of virus.
− Indicates absence of virus.
* Material from two rats pooled.

oculated intranasally with S.L.E. virus and 72 hours later received W.E.E. virus by the same route succumbed to the latter virus. The results obtained when both viruses were instilled into the nose and also when S.L.E. was instilled into the nose and W.E.E. subsequently inoculated intracerebrally suggested that the passage of S.L.E. along the olfactory tract prevented the subsequent passage of W.E.E. over the same tract of 50% of the animals. These results, however, did not explain the manner in which the W.E.E. virus reached the C.N.S. and caused the death of the remaining 50%. Therefore, experiments were conducted in an attempt to determine how W.E.E. virus, following nasal instillation into 3- to 4-week-old rats which had previously received S.L.E. virus intranasally, The brains were divided into two portions, i.e. the anterior rhinencephalon and "remainder of brain." The two anterior rhinencephalons were pooled and a 10% suspension in saline was prepared. The two "remainders of brain" and the two spinal cords were similarly treated. The blood, pool of anterior rhinencephalons, pool of "remainders of brain," and pool of the two spinal cords were tested for the presence of virus by inoculating 0.03 ml of the blood and 0.03 ml of the saline suspensions of the portions of the C.N.S. intracerebrally into 3- to 4-week-old mice and 3- to 4-week-old rats. Both rats and mice were used to test the materials for virus since by the use of these two species one could follow the course of both S.L.E. virus and W.E.E. virus from the nose to the C.N.S. If

virus capable of initiating a fatal infection in mice but not in rats were recovered from the material tested then it could be concluded that S.L.E. virus was present but not W.E.E., since

TABLE 6.—*Tests for W.E.E. in anterior rhinencephalon and "remainder of brain" of rats which received S.L.E. and W.E.E. viruses intranasally.*

Interval following W.E.E. when rats were sacrificed (hours)	Rat no.	Parts tested			
		Anterior rhinencephalon tested for presence of virus in		Remainder of brain tested for presence of virus in	
		Rats	Mice	Rats	Mice
12	1	−	+	−	+
12	2	+	+	−	−
12	3	+	+	−	+
12	4	−	+	−	+
24	5	−	+	−	+
24	6	−	+	−	+
24	7	−	+	−	+
24	8	+	+	−	+
48	9	−	+	−	+
48	10	−	+	−	+
48	11	−	+	−	+

+ Indicates presence of virus.
− Indicates absence of virus.

S.L.E. virus causes a lethal infection in mice but not in rats of this age. On the other hand, if an agent were recovered from the material which would cause a lethal infection in both rats and mice, then W.E.E. was present although S.L.E. virus might also be present. It should be noted that 3- to 4-week-old rats are as susceptible as 3- to 4-week-old mice to intracerebrally inoculated W.E.E. virus,[14] so that if only a small amount of W.E.E. virus were present in the material being tested it could be detected using rats as test animals. It will be seen (table 5) that W.E.E. virus was present in the pool of the anterior rhinencephalons of rats 1 and 2 which were sacrificed 24 hours after inoculation of the W.E.E. virus, since an agent was recovered which induced a lethal infection in rats as well as in mice. W.E.E. virus was not, however, recovered from any other material, although S.L.E. virus was shown to be present in the pool of the "remainders of brain" since the mice inoculated with this material succumbed. By the second day following the inoculation of W.E.E. this virus was found in all parts tested except blood. These findings suggest that if W.E.E. virus, inoculated intranasally 72 hours after S.L.E. virus has been inoculated by the same route, does reach the C.N.S., it does so by way of the olfactory tract since W.E.E. virus can first be detected in the anterior rhinencephalon when it is not present in any other part of the C.N.S. and also when it cannot be recovered from the blood. Since, however, these findings may have been a coincidence, a second experiment was done to obtain more information on this point. Eleven 3- to 4-week-old rats were inoculated intranasally with 0.05 ml of a 10% suspension of S.L.E. virus followed in 72 hours by nasal instillation of 0.05 ml of a 10% suspension of W.E.E. virus. These animals were divided into 3 groups and sacrificed at the intervals indicated in table 6. The anterior rhinencephalons and "remainders of brains" were tested for virus by inoculating a suspension of the part from each rat intracerebrally into two 3- to 4-week-old rats and four 3- to 4-week-old mice. The results are presented in table 6 and show that the W.E.E. virus was recovered from the anterior rhinencephalons of 2 of 4 rats sacrificed 12 hours after they had received W.E.E. intranasally, but that this virus was not recovered from the "remainder of the brain" of any of the 4 animals. W.E.E. virus was recovered from the anterior rhinencephalon of 1 of the 4 rats sacrificed after 24 hours but was not found in the "remainder of brain" of any of the 4 rats. In the case of the 3 rats which were sacrificed 48 hours after they had

14. Duffy, C. J. and Jordan, R. T. Unpublished data.

received W.E.E. virus this virus was not recovered from the C.N.S. The data presented in tables 5 and 6 suggest that in those instances in which W.E.E. virus reaches the C.N.S. of rats, following intranasal inoculation into rats which have previously received S.L.E. by way of the nose, it does so by passing along the olfactory tract since W.E.E. virus can first be recovered from the anterior rhinencephalon when, by the techniques used, it cannot be detected in the remainder of the C.N.S. or in the blood.

DISCUSSION

The data obtained from the experiments reported above indicate that S.L.E. virus, reaching the C.N.S. of rats by way of the olfactory tract, changes the susceptibility of the olfactory nerve to the subsequent invasion of W.E.E. virus. By studying the distribution in rats of W.E.E. virus, which had been instilled intranasally after the rats had previously received S.L.E. virus by the same route, it was found that the W.E.E. virus could be first detected in that part of the C.N.S., i.e., the anterior rhinencephalon, which is located nearest the nasal and olfactory mucosae. This finding suggests that the virus did not circumvent the olfactory nerve and enter the C.N.S. by another nerve pathway, since if this had been the case the virus should have been detected in other parts of the brain at the same time or perhaps before it was recovered from the anterior rhinencephalon. On the other hand, if the W.E.E. virus had first invaded the blood stream from the nasal mucosa and then localized in the C.N.S., it could have in all probability been recovered from other parts of the C.N.S. as early as it was found in the anterior rhinencephalon. Furthermore, W.E.E. virus was not recovered from the blood of the above rats and this observation further suggests that the virus did not reach the C.N.S. by this route.

While S.L.E. virus, invading the C.N.S. of rats by way of the olfactory tract, did not protect all rats against subsequent intranasal inoculation of W.E.E. virus, it did, however, render 50% of the rats resistant and did prolong the incubation period and survival time of those rats which succumbed to infection.

From the data obtained in the studies above no definite conclusions can be drawn regarding the mechanism by which S.L.E. virus, having passed along the olfactory tract, interferes with the subsequent passage of W.E.E. virus over this nerve pathway. However, the anatomy of the nervous system of the rat permits one to postulate that the explanation for the interference observed may be partially on an anatomical basis. In the rat the nerve endings of the olfactory nerve project through the olfactory mucosa. These exposed nerve endings are readily penetrated by such viruses as S.L.E. and W.E.E., providing material containing these viruses is inoculated in such a manner that it is deposited upon the olfactory mucosa. One might assume that in certain rats S.L.E. virus, having been deposited upon the olfactory mucosa, penetrates the endings of all the exposed never fibers and then passes along these fibers to the C.N.S. In those animals in which this occurs the subsequently inoculated W.E.E. virus is completely blocked out and is unable to reach the C.N.S., and these rats, therefore, survive. On the other hand, however, the S.L.E. virus may not penetrate all of the exposed nerve endings and consequently some would be available for invasion by the W.E.E. virus, which could then reach the C.N.S. over a limited number of fibers. This explana-

tion could account for the long incubation period and survival time seen in those rats which do succumb following nasal instillation of both viruses, since the limited number of fibers over which the W.E.E. virus could pass might well reduce the amount of virus which could reach the brain from the olfactory mucosa and, in addition, might prolong the time necessary for it to reach the brain.

SUMMARY

1. St. Louis encephalitis virus instilled into the noses of rats protected 50% of the animals against subsequent infection with Western equine encephalomyelitis virus inoculated by the same route.

2. St. Louis encephalitis virus, following nasal instillation, did not protect rats against Western equine encephalomyelitis virus injected intracerebrally.

3. The data presented suggest that St. Louis encephalitis virus, having progressed from the olfactory mucosa to the central nervous system of rats by the olfactory route, "interferes" with the subsequent passage of Western equine encephalomyelitis virus along this nerve pathway.

INHIBITION OF COMPLEMENT FIXATION BY CERTAIN SERUMS WHEN TESTED AGAINST PSITTACOSIS AND LYMPHO-GRANULOMA VENEREUM ANTIGENS

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During the period of years in which the complement fixation technique has been employed for the detection of antibodies against infectious organisms, workers in this field have cited instances in which certain antiserums of a single individual or of a particular species have possessed inhibitive properties in the complement fixation system. Although these immune serums were produced by repeated injections of the infective organism and possessed high agglutination or precipitation titers, they did not fix complement in the presence of their homologous antigens. They would, however, prevent the fixation of complement by immune serum of another animal or antiserum of another species which had previously been shown to have the power of complement fixation in the presence of their homologous antigens.

In 1910 Calmette and Massol[1] observed that serums from a few calves which had been vaccinated against tuberculosis would precipitate solutions of tuberculin, but did not fix complement in the presence of extracts of the tubercle bacillus. When these immune serums were added to a system containing complement-fixing antiserum produced in a horse and the homologous antigen, the calf immune serum was found to prevent fixation of complement by the horse antiserum and homologous antigen.

Zinsser and Parker[2] in 1923 demonstrated that horse type I pneumococcus antiserum gave powerful precipitation reactions with extracts of type I pneumococcus, but would not fix complement in the presence of the pneumococcus extracts. These workers found that rabbit antipneumococcal serum fixed complement with the homologous pneumococcus antigen, but this fixation could be prevented by adding the horse antipneumococcal serum to the system.

Pittman and Goodner[3] in 1935 described a similar phenomenon which occurred in a system containing the purified, specific carbohydrate of *Hemophilus influenzae* type b and homologous immune serums of rabbits and horses.

In 1936 Goodner and Horsfall[4] made further studies of the inhibition of complement fixation by horse antipneumococcus serum. Experiments were conducted which demonstrated the type specificity of the inhibition reaction. Normal horse serum did not contain the inhibitory substance, nor would heterologous horse immune serum cause the inhibition.

In recent years, Rice[5–7] has demon-

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1. Calmette, A. and Massol, L. 1910, Compt. rend. Soc. de biol. **68**: 224.
2. Zinsser, H. and Parker, J. 1923, J. Immunol. **8**: 151–161.
3. Pittman, M. and Goodner, K. 1935, J. Immunol. **29**: 239.
4. Goodner, K. and Horsfall, F. 1936, J. Exper. Med. **64**: 201–216.
5. Rice, C. E. 1948, Canad. J. Pub. Health, **39**: 72.
6. Rice, C. E. 1948, J. Immunol. **59**: 365.
7. Rice, C. E. 1948, J. Immunol. **60**: 11.

strated that certain avian serums which show high agglutination titers but no complement fixation titers against *Salmonella pullorum* will inhibit complement fixation by rabbit antipullorum serum. The inhibitive powers of the avian serums were found to be related to the agglutination titers. On the basis of these observations, Rice devised a method of titrating the inhibitory activity of antipullorum avian serums and antipneumococcal horse serum. This is called the "indirect" complement fixation test, as it detects not the presence of complement-fixing antibodies, but rather the presence of substances in the serum which inhibit the fixation of complement.

Karrer, Meyer, and Eddie[8,9] applied the indirect complement fixation technique to the diagnosis of ornithosis in chickens and ducks, as serums of these fowls do not give positive reactions in direct complement fixation tests. They have described one pigeon antiserum which showed a prozone in the direct complement fixation test. Dilutions of this serum in which the prozone occurred were found to be inhibitory in the indirect complement fixation test. In one instance a human serum was observed which showed a prozone in the direct complement fixation test against psittacosis antigen. At the time this serum was tested in the indirect complement fixation test for possible inhibitory powers, it has lost its complement-fixing activity. However, this serum was found to be inhibitory in the dilutions in which it had previously shown a prozone in the direct test. Due to the loss of complement-fixing antibodies, this serum could not be shown to possess both inhibitory and complement-fixing activity at the same time.

This paper presents some observations made upon human serums which exhibit a prozone when tested with antigens of the lymphogranuloma venereum-psittacosis group in complement fixation tests. These human serums have been shown to be inhibitory in the complement fixation reaction in the dilutions in which they exhibit a prozone.

MATERIALS AND METHODS

The complement-fixing antigens used in these studies were yolk sac antigens for lymphogranuloma venereum and psittacosis prepared by Markham Laboratories. Control antigens made from normal tissue, as well as positive antiserums for controls, were obtained from Markham Laboratories. In all of the tests, 0.1 ml of a 1:4 dilution of antigen was employed, as recommended by the manufacturer.

Fresh guinea pig serum furnished complement. Two full units in a volume of 0.2 ml were used in all of the reactions.

Sensitized sheep cells were prepared by mixing equal amounts of amboceptor containing two units in a volume of 0.2 ml and 2% washed sheep erythrocytes. In the tests the sensitized cells were used in a volume of 0.4 ml.

Human serums which were known to fix complement in the presence of lymphogranuloma venereum or psittacosis antigens were titrated with the antigens, and one unit, or the highest dilution of serum giving a 4+ fixation with a 1:4 dilution of antigen, was employed in the inhibition test in a volume of 0.2 ml.

The procedure for the direct complement fixation tests was as follows: Serial 2-fold dilutions of the serum to be tested were added to a series of tubes in 0.2 ml amounts. To each dilution of test serum 0.2 ml of complement (two units) and 0.1 ml of a 1:4 dilution of antigen were added. These reagents were incubated at 37 C for 80 minutes, after which 0.4 ml of sensitized sheep cells was added to each tube and incubation continued for an additional 30 minutes at 37 C. The amboceptor and 2% sheep erythrocyte suspension were mixed together 30 minutes previous to being added to the test. Results were reported in terms of 4+ for no hemolysis, 3+ for 25% hemolysis, 2+ for 50% hemolysis, 1+ for 75% hemolysis, and negative for complete hemolysis.

The following procedure was used for the in-

8. Karrer, H., Meyer, K. F. and Eddie, B. 1950, J. Infect. Dis. **87**: 13–23.
9. Karrer, H., Meyer, K. F. and Eddie, B. 1950, J. Infect. Dis. **87**: 24–36.

TABLE 1.—*Method of testing serums for inhibitory properties.*

Tube no.	Test serum dilution	Test serum (ml)	Antigen 1:4 (ml)	One unit of complement-fixing serum (ml)	Complement, 2 units (ml)	*	Sensitized sheep cells (ml)
1	1:2	0.2	0.1	0.2	0.2		0.4
2	1:4	0.2	0.1	0.2	0.2		0.4
3	1:8	0.2	0.1	0.2	0.2		0.4
4	1:16	0.2	0.1	0.2	0.2		0.4
5	1:32	0.2	0.1	0.2	0.2		0.4
6	1:64	0.2	0.1	0.2	0.2		0.4

* Incubated 80 minutes at 37 C.

direct (inhibitory) complement fixation test: Twofold dilutions of the serum to be tested for inhibitory properties were added in 0.2-ml volumes to a series of tubes, and to each tube 0.1 ml of a 1:4 dilution of antigen and 0.2-ml (two units) of complement were added. One unit of positive complement-fixing serum in a volume of 0.2 ml was added to each tube either before the whole mixture was incubated for 80 minutes at 37 C or after incubation of the first 3 reagents for 30 minutes at 37 C. After a total incubation period of 80 minutes at 37 C, 0.4 ml of sensitized cells was added to each tube, and incubation was continued at 37 C for 30 minutes. In these tests, complete hemolysis indicated a positive reaction, i.e., complete inhibition of complement fixation by the antigen and positive complement-fixing serum. Lack of hemolysis indicated a negative reaction, or lack of inhibitory activity on the part of the test serum. Table 1 illustrates the method used for setting up an indirect complement fixation test. It should be noted that the reagents used and quantities of reagents are exactly the same as those in the direct complement fixation test, with the exception of the one unit of complement-fixing serum which is added to each tube in the test. This complement-fixing serum should give 4+ fixation in all of the tubes in the series unless such fixation is inhibited by the test serum.

Direct complement fixation control tests were run on both the inhibitory serum and complement-fixing serum along with anticomplementary controls on all serums and antigens.

EXPERIMENTAL DATA

Among several hundred human serums tested in this laboratory against 14 viral and rickettsial antigens, 54 were positive in dilutions of 1:4 or greater with antigens of the lymphogranuloma venereum-psittacosis group. Of these 54 serums 9 showed a prozone in the initial complement fixation test.

The temperature at which serum is stored prior to being tested seems to have a definite effect upon the manifestation of a prozone in the complement fixation test. Several comparisons were made in which portions of the same serum which had been stored at different temperatures were tested against lymphogranuloma venereum and psittacosis antigens. The results of these comparisons are presented in table 2. Included in this table are 3 of the 9 previously mentioned serums which showed prozones when they were first run in screening complement fixation tests and also 8 additional serums which showed a prozone only after storage at the given temperatures. The length of time these serums were stored ranged from 1 week to 6 weeks. In all instances, a prozone refers to dilutions in which no fixation, or fixation of 1+ or 2+ occurred, followed by fixation of 4+ in higher dilutions of serum.

TABLE 2.—*The effect of storage at different temperatures upon the manifestation of a prozone in human antilymphogranuloma venereum and antipsittacosis serum.*

Serum no.	Highest complement fixing titer	Dilutions of serum in which a prozone occurred after storage at the following temperatures:		
		Room temp., 25 C	Refrigerator temp., 4 C	Deep freeze temp., −50 C
1	1:256	1:2	1:2, 1:4	1:2, 1:4, 1:8
2	1:16	1:2	1:2, 1:4	1:2, 1:4, 1:8
3	1:16	no prozone	1:2	1:2, 1:4
4	1:16	no prozone	no prozone	1:2, 1:4, 1:8
5	1:16	1:2	1:2	1:2, 1:4, 1:8
6	1:32	no prozone	no prozone	1:2, 1:4
7	1:16	no prozone	no prozone	1:2, 1:4
8	1:32	no prozone	no prozone	1:2, 1:4
9	1:8	no prozone	no prozone	1:2, 1:4
10	1:32	no prozone	1:2	1:2, 1:4
11	1:32	no prozone	no prozone	1:2

From the table it may be seen that the prozone occurs to a greater degree in aliquots of serum which have been stored at low temperatures, temperatures of −50 C and lower being most effective in eliciting a prozone reaction. The complement-fixing titer of the serum seems to have no bearing upon the range which the prozone will include, as serums with titers of 1:16 may have prozones as extensive as serums with titers of 1:256. We have also observed in this laboratory that prozones are more prevalent in serums containing complement-fixing antibodies against influenza A and influenza B antigens when these serums are stored in the deep freeze at temperatures of −50 C and lower.

Because prozones occurred in the region of high antibody concentration, it was thought that an excess of antigen in this region might provide a more optimal ratio of antigen and antibody, thereby forming an antigen-antibody complex capable of fixing complement. Table 3 illustrates an example of the results of an attempt to remove the prozone in a serum by the addition of an excess of antigen in the direct complement fixation test. The excess of antigen (two units) seems partially effective in overcoming the prozone.

The possibility was taken into consideration that the inability of certain serums to fix complement in low dilutions with homologous antigen might be due to the presence in this serum of antibodies similar to the "blocking" antibodies which are encountered in Rh agglutination tests.[11] It seemed possible that certain antibodies were present in the serum which combined with the antigen without fixing complement, thereby depriving the complement-fixing antibodies of antigen. Attempts were made to adsorb the inhibitory substance out of the serum by incubating the serum at 37 C with beef albumin in a concentration of 20%. This technique was unsuccessful, and a prozone occurred to the same degree in serums adsorbed with albumin as it did in the same serums which were not adsorbed.

TABLE 3.—*A comparison of the complement fixation reactions of a serum exhibiting a prozone when using one unit of antigen and when using an excess of antigen.*

Serum dilution	Psittacosis antigen diluted 1:4 (1 unit)	Psittacosis antigen diluted 1:2 (2 units)
1:2	—	1+
1:4	—	2+
1:8	—	3+
1:16	—	4+
1:32	4+	4+
1:64	4+	4+
1:128	4+	4+
1:256	4+	4+
1:512	—	—
1:1024	—	—

The prozones encountered in the described serums seemed to resemble the prozones observed in certain pigeon serums and a human serum by Karrer et al.[8,9] These workers have shown that the dilutions of serum within the range of the prozone were inhibitory toward the fixation of complement by anti-psittacosis complement-fixing pigeon serum and psittacosis antigen. Therefore, one of the serums from this laboratory showing an extensive prozone in direct complement fixation tests was tested for its inhibitory properties by the method previously described. Instead of using pigeon serum as standard complement-fixing serum as previous workers had done, one unit of previously tested human antipsittacosis or antilymphogranuloma venereum serum was employed. Table 4 gives the results of direct and indirect complement fixation tests upon a serum showing a prozone.

A comparison of the serum reactions in the two tests reveals that in the dilutions where the serum showed a prozone in the direct complement fixation

test, the serum was inhibitory in the indirect complement fixation test. It should be pointed out that the 4+ reactions in the two highest dilutions of serum in the inhibitory test are due to the presence of the one unit of complement-fixing serum present in all of the tubes in this test. The complement-fixing activity of this serum is, however, inhibited by the lower dilutions of the inhibitory serum, as is evidenced by the lack of fixation in the first tubes in the inhibitory test. The complement-fixing serum was checked at the time of the test by running it in a direct complement fixation test. It was found to show no prozone and to fix complement in all dilutions up to 1:32, the dilution which had been taken as one unit.

These experiments show what the work of Karrer et al[8,9] had not been able to demonstrate—that a single human serum might possess both complement-fixing activity and inhibitory activity in complement fixation systems.

In the above experiment the complement-fixing serum was added to the system along with the inhibitory serum, and both were incubated with complement and antigen. It was found upon further experimentation that the inhibitory power of the test serum was the same regardless of whether the complement-fixing serum was added to the system at the same time as the test serum or was added after the inhibitory serum, antigen, and complement had been in contact for 30 minutes at 37 C. Goodner and Horsfall[4] observed that the secondarily added serum imparted its characteristics to the whole complex. Our results differed from these, because when complement-fixing serum was added 30 minutes after the test serum, it did not impart its character to the complex with resulting fixation. A further experiment was conducted in which the inhibitory serum was added to the system after the complement-fixing serum, antigen, and complement

TABLE 4.—*Reaction of an inhibitory serum in direct and indirect complement fixation tests.*

Serum dilution	Antigens	
	Lympho-granuloma venereum	Psittacosis
Direct test		
1:2	—	—
1:4	—	—
1:8	3+	3+
1:16	4+	4+
1:32	4+	4+
1:64	4+	4+
1:128	4+	4+
1:256	—	—
1:512	—	—
Indirect test		
1:2	—	—
1:4	—	—
1:8	1+	3+
1:16	4+	4+
1:32	4+	4+
1:64	4+	4+
1:128	4+	4+
1:256	4+	4+
1:512	4+	4+

TABLE 5.—*Results of an indirect complement fixation test in which both inhibitory serum and complement-fixing serum were added to the system simultaneously.*

Dilution of inhibitory serum	Antigens	
	Lympho-granuloma venereum	Psittacosis
1:2	—	—
1:4	—	—
1:8	4+	4+
1:16	4+	4+
1:32	4+	4+
1:64	4+	4+
1:128	4+	4+

TABLE 6.—*Results of an indirect complement fixation test in which inhibitory serum was added after thirty minutes incubation of complement-fixing serum, antigen, and complement.*

Dilution of inhibitory serum	Antigens	
	Lympho-granuloma venereum	Psittacosis
1:2	3+	3+
1:4	3+	3+
1:8	4+	4+
1:16	4+	4+
1:32	4+	4+
1:64	4+	4+
1:128	4+	4+

had been in contact for 30 minutes in order to determine if the secondarily added inhibitory serum could impart its characteristics to the system with the inhibition of fixation. Tables 5 and 6 compare the results of this test with the results of a complement fixation inhibition test in which both serums were added at the same time.

In the experiment where inhibitory serum was added after the complement-fixing serum, antigen, and complement had incubated together for 30 minutes at 37 C, it is seen that the inhibitory serum did not impart its inhibitory properties to the whole aggregate, but instead, prevented further fixation from occurring.

From the results of our experiments it seems more likely that the inhibitory effect of the described serums is not due to a rearrangement of the aggregate, but instead, to competition for the antigen by the inhibitory serum and complement-fixing serum.

DISCUSSION

The inhibitory substance in certain immune serums is thought to be antibody, or a substance closely associated with antibody. The evidence for this hypothesis is extensive. The type specificity of the inhibitory substance indicates that it is formed in response to a specific antigen.[4] The power of inhibiting complement fixation is closely correlated with the agglutination and precipitation titers of such serum.[1,2,4,5] Furthermore, an excess of antigen added to a system containing inhibitory antiserum, complement-fixing antiserum, and complement will overcome the inhibitory effects of such serums and cause fixation to occur.[1,5,6,9,10] It seems possible that this excess of antigen furnishes sufficient antigen to combine with the inhibitory antibodies and leaves some free to combine with the complement-fixing antibodies, as is evidenced by positive fixation. In such a system, if an excess of antigen were not available, there presumably would be competition for the antigen by the inhibitory antibodies and the complement-fixing antibodies, with the result that the inhibitory antibodies would not leave enough free antigen to combine with the complement-fixing antibodies. The observation of Karrer et al[9] that one pigeon antiserum might combine with homologous psittacosis antigen with or without fixation of complement lends further support to the assumption that the inhibitory substance is antibody. The inhibitory substance seems to bear some resemblance to the blocking antibodies encountered in Rh typing,[11] and it has been suggested that the inhibitory substance might be a similar type of monovalent antibody which combines with the homologous antigen but forms an aggregate too small to fix complement.[7]

One should also consider the possibility that the inhibitory substance might coat the complement-fixing antibody and prevent its combination with homologous antigen, or at least combination in aggregates large enough to fix complement. It has been demonstrated by Jones and Orcutt[12] that *Brucella abortus* antiserums showing prozones and inhibitory activity in agglutination systems possess specific inhibitory substances which exert their influence by forming a film on the surface of the agglutinin molecules which reduces their cohesiveness and thereby prevents agglutination. Sensitized *Brucella abortus* cells could be freed of this film by wash-

10. Hilleman, M. R., Haig, D. A. and Helmold, R. J. 195, J. Immunol. **66**: 115–130.

11. Wiener, A. 1944, Proc. Soc. Exper. Biol. & Med. **56**: 173–176.

12. Jones, F. S. and Orcutt, M. 1934, J. Immunol. **27**: 215.

ing in saline, and would then agglutinate when resuspended in physiological saline. It was further shown that the inhibitory substance could be adsorbed by homologous bacterial cells. The inhibitory substance is thought to be protein in nature—probably a globulin.

As the inhibitory substance which has been described is generally considered to be antibody-like in nature, the mechanism of its formation and reaction is worthy of speculation. Before this substance was demonstrated in human serum, it seemed to be regarded as being more or less characteristic of certain species which produced this inhibitory substance rather than complement-fixing antibodies in response to contact with the virus. However, with the demonstration of the occurrence of the inhibitory substance in the same species in which complement-fixing antibodies occur, and even in the same serum in which complement-fixing antibodies are present, it becomes evident that inhibitory "antibodies" are not simply a species characteristic.

It seems possible that the substance which combines with specific antigen but does not fix complement might be antibody produced in response to a different antigenic component of the virus than that which elicits the formation of complement-fixing antibodies. This does not, however, explain why storage of serums at cold temperatures should increase the inhibition of complement fixation.

The practical implications of the existence in human serums of inhibitory substances specific for certain diseases must be considered. It has been demonstrated that certain fowls produce only inhibitory antibodies in response to infection with psittacosis viruses. The serums of these birds give negative complement fixation reactions, whereas if they are tested by the indirect complement fixation method, their serums show high inhibitory titers which increase during the course of the disease. It seems possible that certain human cases of psittacosis might produce only inhibitory antibodies, and the infection would not be detected in the routine complement fixation test. Furthermore, serums showing an extensive prozone in the direct complement fixation test might be considered negative if they were tested only in the low dilutions in which the prozone occurred. For this reason it is advisable to test serums in a dilution as high as 1:32 as well as lower dilutions when running routine screening complement fixation tests for viral and rickettsial diseases.

CONCLUSIONS

1. Prozones occur in complement fixation systems when certain human serums are tested against psittacosis and lymphogranuloma venereum antigens.

2. The degree to which a prozone occurs is increased by storing the serum at low temperatures, the most extensive prozones being manifest in serums stored at −50 C.

3. An excess of specific antigen in the complement fixation system will partially remove prozones.

4. Serums which exhibit a prozone in complement fixation tests inhibit complement fixation by a complement-fixing serum and antigen in the dilutions in which they show the prozone.

CHANGES IN NUCLEIC ACIDS AND PROTEIN IN NUCLEATED ERYTHROCYTES INFECTED WITH *PLASMODIUM GALLINACEUM* AS SHOWN BY ULTRAVIOLET ABSORPTION MEASUREMENTS

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In a previous report[1] the author has shown by means of staining techniques and phosphorus fractionations that plasmodia have relatively large amounts of ribose nucleic acid in their cytoplasm. The total nucleic acid content of samples of erythrocytes was shown to vary during the course of the infection. Those samples having a predominance of young, actively growing parasites had a total nucleic acid content that was less than that of uninfected cells even though the ribose nucleic acid fraction of the infected cells was increased. This reduction is assumed to be a reflection of the degradation and loss of desoxyribose nucleic acid from the parasitized host cell nucleus. In cells having a predominance of large multinucleate plasmodia, the desoxyribose nucleic acid fraction was greater than that of the normal, uninfected cell. Since host cell nuclear material was apparently degraded earlier in the course of the infection, this increase was interpreted as being due to an accumulation of desoxyribose nucleic acid as the number of plasmodial nuclei increases. The staining studies which accompanied the phosphorus analyses also suggested changes in the host nuclei, and the latter were occasionally found to be pyknotic and eccentric in position in heavily infected cells. In paludrine-treated infections observations based on staining techniques suggest that parasite nucleic acid may be affected by treatment with the drug. The parasite cytoplasm is pale and less basophilic than normal and nuclear division is abnormal.

The present study was designed to investigate further the changes taking place in the host nucleus and the parasite cytoplasm on an intracellular level, with respect to nucleic acid and protein in treated and untreated infections.

METHODS

The cells studied were from chickens with infections of *Plasmodium gallinaceum* which varied from 2000 parasites/10^4 erythrocytes to 10,000 parasites/10^4 erythrocytes. Slides were prepared early in the individual infection before large numbers of reticulocytes appeared. The paludrine-treated animals were given 100/mg chlorguanide hydrochloride base/kg orally shortly before a segmentation was expected. Slides were made from these animals 30 hours later when effects of the drug on the parasite are readily seen by staining techniques.

The following method of fixation from several tested was used for the measurements presented in this report. Air-dried blood smears were made on quartz slides and placed in an alcoholic lanthanum nitrate solution (15 ml lanthanum nitrate buffer solution at pH 4.68 and 80 ml 95% ethyl alcohol). The lanthanum mixture is one of

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1. Lewert, R. M. 1952, Nucleic acids in plasmodia and the phosphorus partition of cells infected with *Plasmodium gallinaceum*. J. Infect. Dis. **91**: 125–144.

those described by Hammarsten.[2] Slides were fixed for 30 minutes to several hours. Immediately before examination the mixture was replaced by glycerine containing a small amount of lanthanum nitrate as the mounting medium and the smear was covered with a quartz coverslip. Air-dried smears were used in the present studies in preference to wet-fixed preparations to obtain an essentially uniform thickness of the erythrocyte nucleus.

Absorption measurements were made with a quartz microscope with the apparatus arranged as described by Catchpole and Gersh,[3] using a 48× reflecting objective. Measurements were made of a cylinder about 1 μ in diameter through the nucleus and cytoplasm of each cell at 2652 Å and 2804 Å near the maximum absorption peaks of nucleotides and cyclic amino acids, respectively. In infected cells, measurements were made through the parasitized area. Parasites were located and the degree of infection of individual cells was determined by observing the field with a "finder" containing a fluorescent screen.

RESULTS

The results are presented in tabular form as extinctions with standard errors. Measurements of the cells are roughly grouped according to the degree of infection. Where differences in means are compared in the results, the *P* values have been computed and are included in the table. Cells with small parasites and with the erythrocyte nucleus in normal position may be considered lightly infected. Cells containing large parasites and having an eccentric nucleus may be considered heavily infected cells.

In heavily infected cells the host cell nuclear absorption at the nucleotide peak is approximately half that of the normal erythrocyte nucleus. Nuclei of cells containing small parasites have an absorption intermediate between that of normal and heavily infected cells. The nuclei of paludrine-treated, infected cells have absorptions similar to those found in the lightly infected, untreated cells.

Changes in nuclear protein are shown by the measurements made at 2804 Å. This portion of the host nucleus decreases in all infected cells roughly in proportion to the degree of infection. In this respect erythrocyte nuclei of paludrine-treated, parasitized cells are similar to the nuclei of the cells from the untreated infection.

An estimate of the comparative decrease of nucleotides and amino acids can be made from column $\frac{E\ 2652}{E\ 2804}$ of the table. It is apparent that the nucleotides are degraded at a greater rate than the protein portion of the infected nucleus, whereas the $\frac{E\ 2652}{E\ 2804}$ ratio is essentially the same as that of the normal cell in the paludrine-treated infections.

The changes in the parasite cytoplasm at 2652 Å and 2804 Å are not generally as extensive as those found in the erythrocyte nuclei. They indicate that there is a greater quantity of nucleotides in the cytoplasm of the smaller parasites as compared to the larger parasites. Nucleotides significantly decrease in the cytoplasm of the parasites from the paludrine-treated series. The protein absorption measurements show that the young parasites have a greater absorption than the older parasites and that the large parasites in the paludrine-treated infection have less absorption than either of these groups. Using the absorption of the normal erythrocyte

2. Hammarsten, E. and Teorell, T. 1928, Versuche über mikrochemische Reactionen. II. Ausfällungen von Eiweiss-nucleinsaure mit Lanthan- und Sulfosalizylsaureionen. Acta med. Scandinav. **68**: 219–238.
3. Catchpole, H. R. and Gersh, I. 1950, Components of normal and abnormal liver cells studied by ultra-violet microscopy and by differential centrifugation. Faraday Soc. Disc. **9**: 471–480.

as a base line for protein absorption, the comparative rate of decrease, $\frac{E\ 2652}{E\ 2804}$ of table, shows that the nucleotide to amino acid ratio is slightly less than normal in the untreated, infected cells and essentially the same as normal in the paludrine-treated series.

DISCUSSION

The loss of nucleotides from the host nucleus of infected cells demonstrated by the ultraviolet absorption measurements was anticipated from the previous studies of cells by phosphorus fractionation. Not only is transmission at 2652 Å greatly increased in such cells, but there is also a nuclear volumetric decrease that averages almost 40%. In less heavily infected cells the decrease in nuclear volume averages about 30%. The volume decrease itself does not materially affect the transmission measurements since the thickness of the central area of the nucleus was comparable on the air dried smears. If both increased transmission and decreased volume are considered, the total nucleotide loss is probably in excess of 50% in heavily infected cells.

In the paludrine-treated series, the loss of host cell nucleotides as shown by transmission measurements is about the same as that found in the lightly infected untreated cells, but the volume decrease of the nucleus is only 10 to 20% as compared to 30% in lightly infected, untreated cells. Therefore, the degradation of host nuclear material in the heavily infected, paludrine-treated cell is probably less than or of similar magnitude to that occurring in the cell containing a small parasite not exposed to paludrine. The nucleus of the infected cell in both the treated and untreated series loses similar amounts of protein material or at least those cyclic amino acids that account for the 2804 Å absorption peak. Since the paludrine-treated series lose less nucleotide material than does the untreated series, the ratio of nucleotide to amino acid remains the same as that of the normal cell. In untreated, infected cells the ratio indicates a greater proportionate loss of nucleotides.

Absorption measurements of the cytoplasm of the parasites reveal interesting differences that may be correlated with observations made on stained preparations. The cytoplasm of large,

TABLE 1.—*Average extinction coefficient in normal, infected, and paludrine-treated infected chicken erythrocytes.*

Group no.	Cell type	Number of determinations	Erythrocyte nucleus Average extinction coefficient		Ratio $\frac{E2652}{E2804}$	Erythrocyte and parasite cytoplasm Average extinction coefficient		Ratio $\frac{E2652}{E2804}$
			Nucleotide peak (2652 Å)	Protein peak (2804 Å)		Nucleotide peak (2652 Å)	Protein peak (2804 Å)	
1	Normal	50	0.703±0.008	0.533±0.008	1.32	0.272±0.006	0.203±0.004	1.34
2	Infected, nuclei displaced	25	0.467±0.023	0.430±0.014	1.09	0.255±0.015	0.214±0.012	1.19
3	Infected, nucleus normal in position	25	0.599±0.018	0.490±0.015	1.22	0.320±0.003	0.251±0.003	1.27
4	Paludrine-treated infected, nuclei displaced	25	0.596±0.017	0.442±0.014	1.35	0.191±0.011	0.143±0.011	1.33
	Significance of difference between means		1&2, $P<0.001$ 2&4, $P<0.001$	1&2, $P<0.001$		2&3, $P<0.001$ 2&4, $P<0.001$	2&4, $P<0.001$	

untreated parasites does not differ markedly from that of the host cell with respect to either nucleotide or amino acid absorption. In small parasites, whose cytoplasm is more basophilic, there is a slightly increased absorption of both wave lengths. At certain stages of the parasite in stained material, interpreted as a period of growth between nuclear division, the cytoplasm is very intensely stained and is basophilic or metachromatic with some techniques.[1] This basophilia is completely removed by ribonuclease. Such stages are probably represented in the present study by parasites whose absorption at 2652 Å was as great as or greater than that of the host nucleus. These particular measurements are interpreted as indicating concentrations of ribose nucleic acid in the parasite cytoplasm. The protein absorption measurements of the parasite are slightly greater than those of the normal erythrocyte cytoplasm, indicating an increase in protein. This finding agrees with the work of Groman,[4] who has shown increase in the amino nitrogen infected cells although total nitrogen remains unchanged.

It is further interesting to note that the staining characteristics of paludrine-treated plasmodia can also be correlated with the absorption measurements of these cells. After such treatment the stained parasites have a pale cytoplasm which is less basophilic than normal. Absorption measurements of these large paludrine-treated parasites show not only a lower level of nucleotides in the parasite but also a decrease in protein as compared to untreated parasites of similar size. Other authors[5] have shown that paludrine inhibition of the growth of *Lactobacillus casei* is reversed by purines and pteroylglutamic acid and have suggested that this relationship may be involved in the systems affected by antimalarials. Greenberg[6] has shown more directly inhibition of antimalarial activity of paludrine by pteroylglutamic acid in *P. gallinaceum* infections. The present data also suggest that paludrine interferes with nucleic acid synthesis by the parasite and with the degradation and possible utilization of nucleic acid of the host cell nuclei.

4. Groman, N. B. 1951, Dynamic aspect of the nitrogen metabolism of *Plasmodium gallinaceum* in vivo and in vitro. J. Infect. Dis. **88**: 126–150.

SUMMARY

Ultraviolet absorption measurements indicate that plasmodia in nucleated erythrocytes cause a degradation of both the nucleic acid and protein constituents of the erythrocyte nucleus. Nucleotides are lost at a greater rate than amino acids, and as much as 50% or more of the former may be removed. Paludrine treatment of infected cells interferes with the degradation and loss of host nuclear material, particularly with respect to nucleic acid. The loss of amino acids is apparently not affected by paludrine treatment or is affected to a much lesser extent. In addition, paludrine treatment interferes with the accumulation of ribose nucleic acid in the parasite cytoplasm as well as with the production of cytoplasmic protein by the parasite.

5. Falco, E. A., Hitchings, G. H., Russel, P. B. and VanderWerff, H. 1949, Antimalarials as antagonists of purines and pteroylglutamic acid. Nature, **164**: 107–108.

6. Greenberg, J. 1949, Inhibition of the antimalarial activity of chlorguanide by pteroylglutamic acid. Proc. Soc. Exper. Biol. & Med. **71**: 306–308.

THE EFFECT OF TRYPSIN AND OTHER ENZYMES ON FELINE PNEUMONITIS VIRUS

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Feline pneumonitis virus obtained from chick embryo yolk sac (Moulder and Weiss, 1951) was treated with a variety of purified enzymes in the hope that knowledge of its susceptibility to enzymic attack would help in defining the chemical structure of the virus surface and in separating the virus from normal yolk sac components. Like many other viruses already studied, feline pneumonitis virus was not inactivated or otherwise detectably altered by proteinases, nucleases or lipases. However, the proteinases significantly increased the infectivity of the purified virus, probably by decreasing the degree of aggregation of the elementary bodies. Since the proteinases also reduce large amounts of normal yolk sac structures to nonsedimentable dimensions, the use of a proteinase such as trypsin in the purification of feline pneumonitis virus from yolk sac increases the yield and purity of the separated virus, while at the same time simplifying the preparative procedure.

MATERIALS AND METHODS

The strain of feline pneumonitis virus, its propagation in the yolk sac of chick embryos, the titration of virus infectivity in terms of the LD_{50} for chick embryos, the modified Gram stain for elementary bodies and the phosphate-saline buffer used in preparing and diluting the virus have been described by Moulder and Weiss (1951). Total nitrogen was measured by the micro-Kjeldahl method of Groman (1951).

Purification of the virus for enzymic studies.—The procedure for purifying feline pneumonitis virus for the enzymic studies will not be described in detail because a simpler and more efficient method of purification has now been devised and will be described separately. The virus was purified from yolk sac by a procedure similar to that given by Moulder and Weiss (1951) except that normal yolk sac material was flocculated with bovine serum albumin (Bovarnick and Miller, 1950) before the preparation was treated with antiserum against normal yolk sac. This procedure yielded a final product containing about 10% of the original infectivity. One LD_{50} was equal to $10^{-11.2}$ g total nitrogen. Such virus was used for investigating the action of the proteinases. When the virus was digested with trypsin after immune precipitation of normal components as described in a later section, the recovery of infectivity was as high as 30%, and one LD_{50} was contained in $10^{-12.0}$ g total nitrogen. The trypsin-treated virus was used for most of the enzyme studies other than those with proteinases.

Source of enzymes and assay of enzymic activity.—Table 1 gives the source of the enzymes tested on feline pneumonitis virus and the way in which their activity was assayed. The activity of the crystalline enzymes was close to the reported values. The activity of the partially purified enzymes is listed in table 1. Each enzyme attacked appropriate known substrates under the same conditions used for study of its effect on the virus and high concentrations of enzyme were always employed. Therefore, it is unlikely that the failure of a given enzyme to attack the virus was due to unfavorable conditions for activity or to inadequate concentration. The enzyme-virus mixtures were diluted at least 10^4 times before inoculation into the chick embryos. At these concentrations, none of the enzymes had any effect upon development of the embryos.

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TABLE 1.—*Description of enzymes and methods of assay.*

Enzyme	Description	Method of assay
Trypsin	Crystallized and recrystallized products from Armour Laboratories	Digestion of hemoglobin, Anson (1938)
Chymotrypsin	Recrystallized, Armour Laboratories	Digestion of hemoglobin, Anson (1938)
Pepsin	Crystallized, Armour Laboratories	Digestion of hemoglobin, Anson (1938)
Papain	Crude papain (Syn-Zyme Laboratories) repeatedly precipitated at 0.4 saturation with ammonium sulfate. 0.1 unit activity per mg protein nitrogen with no added activator	Digestion of hemoglobin, Anson (1938)
Lecithinase	Concentrated and lyophilized filtrate from a culture of *Clostridium welchii*, released 200 μg P per mg dry weight per hour at 25 C*	Release of acid-soluble phosphorus, MacFarlane and Knight (1941)
Lipase	Purified from *Fusarium lini* Bolley by Fiore and Nord (1950), stage 5, released 0.04 mM fatty acid per hour at 25 C under Archibald's conditions†	Hydrolysis of Tween 20, Archibald (1946)
Ribonuclease	Crystallized, Armour Laboratories	Manometric measurement of liberated acid groups, Bain and Rusch (1944)
Desoxyribonuclease	Crystallized, Syn-Zyme Laboratories, Worthington Laboratories	Digestion of chicken erythrocyte nucleus, Lewert (1952)
Hyaluronidase	Partially purified from bull testes, 184 units per mg‡	Turbidimetric measurement of hyaluronic acid hydrolysis, Dorfman and Ott (1948)

* The gift of Dr. William Burrows, Dept. of Bacteriology and Parasitology, the University of Chicago.
† Generously supplied by Dr. F. F. Nord, Department of Chemistry, Fordham University, N. Y.
‡ The gift of Dr. E. P. Benditt, Dept. of Pathology, the University of Chicago.

EXPERIMENTAL RESULTS

The effect of the different enzymes on feline pneumonitis virus was tested in the same general manner. To study the effect of an enzyme on virus infectivity, purified virus suspensions which had been stored in sealed Pyrex glass tubes at −70 C for not more than a month were quickly thawed, diluted in phosphate-saline, pH 7.4, to a concentration of about 0.02 mg total nitrogen per ml ($10^{6.5}$ LD_{50} per ml) and held at 0 C. One sample of the virus suspension was immediately inoculated into chick embryos for determination of the initial infectivity titer. A second sample was mixed with an equal volume of phosphate-saline and incubated at 25 C, while a third sample was mixed with an equal volume of the enzyme dissolved in phosphate-saline and also incubated at 25 C. After incubating at 25 C for the desired time, the enzyme-treated virus and the virus incubated with phosphate-saline alone were chilled to 0 C and their infectivity titrated in chick embryos. The difference in infectivity between the first and second samples gave the loss of infectivity at 25 C in the absence of the enzyme. Figure 1 shows that this loss was appreciable even for

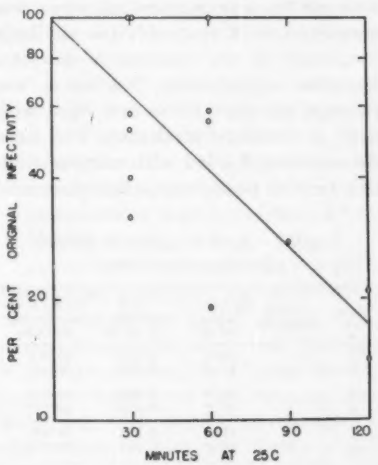

FIG. 1.—The stability of purified feline pneumonitis virus in phosphate-saline at pH 7.4 and 25 C. The straight line was fitted to the data of 14 independent experiments by the method of least squares.

brief incubation at 25 C. The decline in infectivity appears to follow first order reaction kinetics and the half-life of infective virus in phosphate-saline at pH 7.4 and 25 C is about 50 minutes. Therefore, the effect of the enzyme on the active virus was taken as the difference in infectivity between samples 2 and 3.

The ability of each enzyme to attack killed virus was studied by examining microscopically the enzyme-treated elementary bodies for changes in size, shape and staining properties. When purified virus was inactivated by heating at 100 C for 15 minutes, the elementary bodies were still stained by Gram stain when the period of alcohol decolorization was less than 5 seconds (Moulder and Weiss, 1951) and were of normal appearance, although strongly clumped. The various enzymes were added to the heat-inactivated virus and incubated at 25 or 37 C for as long as 24 hours. The microscopic appearance of the enzyme-treated virus was compared with that of virus similarly incubated in the absence of enzyme. In other experiments, the virus was smeared on glass slides and fixed with heat or absolute methanol. The fixed smears were flooded with enzyme solution or with phosphate-saline alone and kept in a moist chamber at 30 C for up to 24 hours before they were stained and examined. Although these procedures are much less sensitive indicators of enzymic attack than titration of infectivity, they should certainly detect any gross disintegration of the elementary bodies.

TABLE 2.—*Effect of trypsin on purified feline pneumonitis virus.*

Exp. no.	Tryptic digestion	Infectivity LD_{50} per ml	Total nitrogen, mg per ml	Grams of total nitrogen per LD_{50}
1	−	$10^{8.6}$	0.033	$10^{-11.2}$
1	+	$10^{7.0}$	0.027	$10^{-11.6}$
2	−	$10^{8.0}$	0.030	$10^{-11.2}$
2	+	$10^{7.0}$	0.020	$10^{-11.7}$
3	−	$10^{7.0}$	0.055	$10^{-11.3}$
3	+	$10^{7.7}$	0.042	$10^{-11.6}$

A sample of virus in phosphate-saline, purified as described in *Methods and Materials*, was divided into two fractions. One was incubated 30 minutes at 25 C with 0.5 mg recrystallized trypsin per ml, while the other was incubated without enzyme addition. Both fractions were then sedimented by centrifugation in an angle centrifuge at 3000 ×*g* for 1 hour at 0 C. The sediment was washed once by centrifugation and suspended to the original volume in phosphate-saline.

The action of proteinases

Effect of proteinases on virus infectivity.—Hoagland, Ward, Smadel and Rivers (1940) have shown that vaccinia virus treated with papain loses its infectivity and characteristic shape and liberates free amino nitrogen, and Bawden and Pirie (1936) have reported that potato X virus is inactivated by trypsin. However, most native viruses have resisted hydrolysis by proteinases, and tryptic digestion of host protein has been used in the purification of several viruses (Smadel and Wall, 1937; Warren, 1950).

When purified feline pneumonitis virus was incubated in 0.025 to 0.25 mg recrystallized trypsin per ml for 30 to 60 minutes at 25 C, the trypsin-treated virus always had a much higher infectivity than did untreated virus. The effect of trypsin was more marked with old suspensions which had suffered great loss of infectivity than with fresh virus suspensions. When purified virus was frozen and stored at −70 C until its LD_{50} for chick embryos was sharply reduced, digestion with 0.25 mg trypsin per ml for 30 minutes at 25 C increased its infectivity 10 times, almost to the level originally possessed by the fresh virus. The LD_{50} of the fresh, unfrozen virus was increased 2 to 4 times by similar treatment (table 2). The infectivity of crude emulsions of yolk sac infected with feline pneumonitis virus was also raised 2 to 4 times when they were digested with 1 to 3 mg trypsin per ml for 1 hour at 25 C (table 3). The higher

trypsin concentration required to increase the infectivity of crude emulsions is probably due to their greater protein content.

Several lines of evidence suggest that the infectivity-increasing effect of trypsin is the result of its proteolytic activity. (1) The action of trypsin is shared by the proteinases chymotrypsin and papain. When tested in the same way as trypsin, they both produce a similar increase in the LD_{50} of purified virus. (2) The crystalline trypsin inhibitor from soy beans (Armour Laboratories) inhibits the infectivity-increasing action of trypsin in the same concentration necessary to inhibit hydrolysis of hemoglobin in the trypsin assay system (Anson, 1938). (3) Trypsin produces a small but consistent decrease in the sedimentable nitrogen of purified virus suspensions (table 2). (4) Once the virus has been exposed to any one of the three proteinases, a second digestion with either of the other two does not further increase virus infectivity.

A plausible explanation for the way in which trypsin increases the infectivity of feline pneumonitis virus is that it hydrolyzes a layer of protein on the surface of the elementary bodies which is not required for full infectivity but which greatly increases the tendency of the elementary bodies to aggregate. Therefore, digestion of the protein would tend to disperse the elementary bodies and thus increase the number of independent infective units. This postulated mechanism for tryptic action has experimental support, although much of it is indirect.

First, the elementary bodies of feline pneumonitis often exist in a highly aggregated state. The virus is present in infected cells mainly in the form of vesicles, which appear to consist of large masses of elementary bodies (Weiss, 1949). Electron micrographs of crude virus preparation often show the elementary bodies embedded in a matrix of unknown origin (Hamre, Rake and Rake, 1947; Moulder and Weiss, 1951), while dark-field examination of purified virus suspensions showed that clumps of up to 50 elementary bodies were present, particularly at high virus concentrations.

Second, the degree of aggregation of a virus suspension was correlated with its infectivity and with the magnitude of the tryptic effect. Strongly aggregated preparations had low infectivities which were markedly increased by trypsin. A rough semiquantitative estimate of the degree of aggregation could be obtained by counting with a dark-field microscope and a Petroff-Hauser chamber the number of clumps of an arbitrary size per ml. Trypsin reduced the aggregation of the elementary bodies by about 50%. Although the reduction was not dramatic, it was consistent, and of a variety of agents tested, only trypsin, chymotrypsin and papain were effective.

It is unlikely that trypsin increases virus infectivity by destroying inactive virus because no interference with virus multiplication in chick embryos occurred when ultra-violet inactivated virus was added to active virus in a ratio of 100,000 to 1.

Use of trypsin in purification of feline pneumonitis virus from yolk sacs.—Tryptic digestion has aided in preparing feline pneumonitis virus from yolk sacs in greater yield and in a higher state of purity. Trypsin was first added to virus which had been purified as described in the section on methods. Table 2 indicates that a tryptic digestion at this point increased the LD_{50} of the preparation two- to fourfold and slightly decreased the total nitrogen content. The ratio of infectivity to total nitrogen was

significantly higher after trypsin treatment.

More recently, comparatively high concentrations of crystallized trypsin were added directly to crude infected yolk sac emulsions (table 3). When the virus was centrifuged down after incubating with trypsin for 1 hour at 25 C, the sediment contained 3 times as much infectivity and only about one-

TABLE 3.—*Effect of trypsin on feline pneumonitis virus in crude yolk sac emulsions.*

Fraction	Infectivity LD_{50} per ml	Total nitrogen, mg per ml	Grams of total nitrogen per LD_{50}
Original homogenate	$10^{8.70}$	3.800	$10^{-9.13}$
Original homogenate after tryptic digestion	$10^{7.22}$	3.800*	$10^{-8.44}$
Sedimented without tryptic digestion	$10^{8.62}$	0.562	$10^{-9.77}$
Sedimented after tryptic digestion	$10^{8.88}$	0.150	$10^{-10.00}$

An emulsion of infected yolk sacs in twice their weight of 0.1 *M* phosphate, pH 7.4, was homogenized 30 seconds in a Waring blendor. One-half was incubated with 2 mg crystallized trypsin per ml for 1 hour at 25 C and the other was incubated without addition of enzyme. Both were sedimented in an angle centrifuge at 0 C for 1 hour at 5000 × *g*. The supernates were discarded and the sediments were suspended to the original volume in phosphate-saline.
* Exclusive of trypsin nitrogen.

fourth the total nitrogen as a comparable undigested sample. The yolk sac lipids were also much more easily removed after tryptic digestion, possibly because of the disruption of lipoproteins. Feline pneumonitis virus may be separated from such trypsin-digested yolk sac emulsions in high yield and with a high ratio of infectivity to total nitrogen by a rapid and simple procedure to be described separately.

Effect of proteinases on inactivated virus.—Since many proteins are more easily hydrolyzed by proteinases in the denatured than in the native state (Neurath et al, 1944), inactivated virus was treated with trypsin, chymotrypsin and papain in concentrations of 0.5 to 1.0 mg per ml, but there was no microscopically observable digestion of the elementary bodies even after prolonged contact with the enzymes.

Pepsin attacks tobacco mosaic virus (Stanley, 1934), vaccinia virus (Hoagland et al, 1940; Dawson and MacFarland, 1948), and potato virus X (Bawden and Pirie, 1936; Kleczkowski, 1944). To test the action of pepsin, active feline pneumonitis virus was centrifuged down, suspended in 0.01 *N* hydrochloric acid, centrifuged down again, and resuspended in 0.01 *N* hydrochloric acid. At this pH, the virus was quickly inactivated. Pepsin in 0.01 *N* hydrochloric acid was added to a final concentration of 1 mg per ml. After 24 hours at 37 C, there was no significant change in the microscopic appearance of the pepsin-treated virus as compared to virus incubated in 0.01 *N* hydrochloric acid alone.

The action of other enzymes and agents

Effect of lipolytic enzymes.—Two fat-splitting enzymes were tested on feline pneumonitis virus, the lecithinase of *Clostridium welchii* and the lipase of *Fusarium lini* Bolley. The lecithinase hydrolyzes lecithin to phosphocholine and a diglyceride (MacFarlane and Knight, 1941), while the lipase liberates fatty acids from neutral fat (Fiore and Nord, 1950). Since lecithin requires calcium ion as an activator, its action was tested in 0.01 *M* histidine, pH 7.3, and 0.002 *M* calcium chloride. When virus which had been purified by the procedure including tryptic digestion was incubated for 1 hour at 25 C with 5 mg per ml lecithinase or lipase, there was no change in infectivity or microscopic appearance of the virus. A 30-minute incubation at 25 C with 0.5 mg per ml of either trypsin or papain had no effect on virus already digested with lecithinase or lipase. Inactivated virus was not visibly attacked by either of the two enzymes separately in 24 hours at 25 C, or when this treatment was fol-

lowed by digestion with papain or trypsin.

Effect of nucleases.—Although all viruses studied contain either ribonucleic or desoxyribonucleic acid, the nucleic acids of intact viruses are not broken down by either ribonuclease or desoxyribonuclease. Dawson and McFarlane (1948) have found that vaccinia virus contains structures destroyed by desoxyribonuclease, but only after the virus has been digested with pepsin. Among bacteria, ribonuclease destroys the Gram-positive reaction of some Gram-positive bacteria, apparently by hydrolyzing ribonucleic acid located on the cell surface and essential for Gram staining (Bartholomew and Umbreit, 1944).

In a concentration of 1 mg per ml, neither ribonuclease nor desoxyribonuclease had any effect on the infectivity of feline pneumonitis virus in 1 hour at 25 C. Desoxyribonuclease did not change the microscopic appearance or staining properties of either active or inactive virus. To learn if ribonuclease could destroy the weak Gram-positive staining of the virus, trypsin-treated virus and an 18-hour culture of *Staphylococcus aureus* were inactivated by heating and treated with ribonuclease at 45 C as described by Bartholomew and Umbreit (1944), except that the decolorization with 95% ethanol was limited to 5 seconds. Most of the staphylococci became Gram-negative after 1 hour at 45 C, but the virus remained Gram-positive at the end of 48 hours. This experiment suggests that the basis of the Gram-positive reaction in the virus is not the same as in bacteria.

The effect of hyaluronidase.—Hyaluronidase hydrolyzes the mucopolysaccharide, hyaluronic acid, which occurs in several microorganisms, and was the only carbohydrase studied. In a concentration of 1 mg per ml it had no effect on virus infectivity or morphology after 1 hour at 25 C. Inactivated virus was also not visibly attacked.

Effect of surface-active agents and ether.—Since feline pneumonitis virus was resistant to a variety of enzymes, the effect of potential inactivators of less specific modes of action was next studied.

Surface-active compounds are well known bactericidal and viricidal agents. Their effectiveness appears to lie in their ability to combine with the proteins of the organism affected (Putnam, 1948). Both the cationic surface-active agent, cetyl pyridinium chloride,* and the anionic agent, sodium dodecyl sulfate, inactivated the virus, but the cationic agent was much more effective (table 4). The surface-active agents destroyed the infectivity of the elementary bodies without apparent changes in morphology as observed microscopically.

TABLE 4.—*Effect of surface-active agents on purified feline pneumonitis virus.*

Agent	Concentration, mg per ml	Degree of inactivation
Cetyl pyridinium chloride	1.0	Complete
Cetyl pyridinium chloride	0.1	Complete
Cetyl pyridinium chloride	0.01	Partial
Sodium dodecyl sulfate	1.0	Complete
Sodium dodecyl sulfate	0.1	None

Virus purified by tryptic digestion was incubated with the surface-active agents for 30 minutes at 25 C. The concentration of virus was 0.003 mg total nitrogen per ml.

Members of the psittacosis-lymphogranuloma group have been uniformly reported to be inactivated by diethyl ether. Feline pneumonitis virus was also ether-sensitive. Contact with peroxide-free ether for 15 minutes at 0 C destroyed more than 99% of its infectivity.

* Very pure samples of these compounds were generously donated by Dr. F. W. Putnam of the Department of Biochemistry.

DISCUSSION

The great resistance of the elementary bodies of feline pneumonitis to enzymic attack contrasts sharply with the ease with which they are inactivated by heat, extremes of pH (Moulder and Weiss, 1951), surface-active agents and ether. It is probable that the virus is inert toward enzymes because the proper specific chemical groups necessary for enzyme-substrate combination are not present on the surface of the elementary bodies and that such specific groups are not exposed by inactivation of the virus. However, substances, such as the surface-active agents, which are less specific in their requirements for combination, are able to react with the virus surface in such a way as to make the virus noninfective.

SUMMARY

Treatment of both crude and purified feline pneumonitis virus from yolk sacs with trypsin raises its infectivity for chick embryos two- to tenfold. This effect is also produced by chymotrypsin and papain. The proteinases apparently act by dispersing aggregates of elementary bodies, thus increasing the number of independent infectious units. Tryptic digestion is of value in purifying the virus from yolk sac. Pepsin did not visibly digest the virus.

Neither active nor inactive virus was attacked by *Clostridium welchii* lecithinase, *Fusarium lini* Bolley lipase, ribonuclease, desoxyribonuclease or hyaluronidase, alone or in various combinations.

Feline pneumonitis virus was inactivated by cetyl pyridinium chloride, sodium dodecyl sulfate and diethyl ether.

It was concluded that the virus resists enzymic attack because the chemical groupings necessary for enzyme-substrate combination are not present on its surface.

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DEMONSTRATION OF AN ENZYMATIC FACTOR IN CERCARIAE OF *SCHISTOSOMA MANSONI* BY THE STREPTOCOCCAL DECAPSULATION TEST

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It has long been postulated that schistosome cercariae penetrate the skin of their definitive hosts with the aid of a histolytic enzyme. Evidence of such a lytic agent in the cercariae of *Schistosoma mansoni*, similar in action to the "spreading factor" of Duran-Reynals (1942), was noted by Kuntz in unpublished experiments. He found that the diffusion of india ink mixed with suspensions of ground cercariae and injected into the skin of rabbits was greater than that of India ink-saline controls.

Hyaluronidase has been reported by Levine et al (1948) on the basis of results of viscosimetric assay of ground cercariae. However, by this method, no reduction in viscosity of hyaluronic acid could be shown without previous incubation of the cercarial suspension on sodium hyaluronate at 25 C for 18 hours. Since the bacteria present in controls of snail feces maintained under similar conditions also showed no viscosity reducing activity, it may be that the viscosimetric method is not sensitive enough to assay separately the hyaluronidase production either of the bacteria or of the cercariae alone. It was shown by McClean (1941) that actively growing bacteria increase their hyaluronidase production markedly when incubated on hyaluronate, but this fact can hardly be extrapolated to include free-living schistosome cercariae. The possibility, then, that the results of Levine et al reflect the sum of such induced bacterial enzyme production plus that of the cercariae cannot be completely eliminated. If this is the case, it is not surprising that no correlation could be demonstrated by viscosimetric methods between the number of cercariae present and the rate of viscosity reduction.

Our projected studies required a method of assay sensitive enough to detect the enzyme present in unincubated suspensions of cercariae of *S. mansoni* and in bacterial controls alone, permitting concurrent check on enzyme production by bacteria unavoidably present in the cercarial suspensions and showing correlation of enzymatic activity with the number of cercariae employed.

A test which fulfills these requirements employs the capsules of group C mucoid β hemolytic streptococci as the substrate. The observations of Seastone (1939), confirmed by many investigators whose work was reviewed by Morison (1941), show that one of the principal components of the capsular material of these streptococci is

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hyaluronic acid. While the structural relationship of this constituent to the organism is unknown, it has been repeatedly demonstrated that enzymes of the hyaluronidase complex depolymerize and subsequently hydrolyze the substrate, causing the capsule to diffuse into the surrounding medium with resultant disappearance of the capsular zone. Of especial value for this study is the observation of Fulton, Marcus, and Robinson (1948) that the streptococcal decapsulation test, with their techniques, offers a method for the quantitative assay of dried testicular hyaluronidase sensitive to 0.001 to 0.0001 viscosity reducing unit per ml.

This investigation was designed, therefore, to demonstrate qualitatively the effect of an enzyme or enzyme system in cercariae of *S. mansoni* on the specific substrate material accreted by encaplated streptococci.

MATERIALS AND METHODS

The streptococci employed were generously supplied by Dr. C. V. Seastone of the Department of Medical Microbiology of the University of Wisconsin. They were of his strain M4, which is a group C strain from guinea pig lymphadenitis. The organisms were maintained by daily subculture on human blood agar plates, pH 7.2. For the substrate, a relatively uniform suspension was obtained by transfer of 10 to 12 isolated colonies from a 20-hour blood agar plate culture to a conical centrifuge tube containing 5 ml difco brain-heart infusion enriched with 1 ml human plasma and cultured at 35 C for 4 hours. The cultures were shaken vigorously every hour to prevent long chain formation. At the end of this period, they were centrifuged at high speed for 10 minutes, the streptococci resuspended in 5 ml of either distilled water or 0.1 *M* phosphate buffer, pH 6.0, recentrifuged, and the final substrate obtained by resuspending the organisms in 1 ml of the diluent.

The infected snails (*Australorbis glabratus* Say) were washed through four changes of fresh distilled water, removing most of the adherent fauna, transferred to gum evaporating dishes containing enough water to cover the snails, and placed in a well-lighted warming oven at 35 C to insure maximum cercarial emergence. After two hours the snails were returned to their culture dishes and the cercariae separated from the fecal material by alternately allowing the feces to settle out and carefully decanting the supernatant cercarial suspension until all visible particles were removed.

The cercarial suspension thus obtained was transferred to a 250 ml settling glass, placed in the refrigerator, and the larvae allowed to settle out for a period of 4 hours at 8 C. The cercaria-free supernatant fluid was then withdrawn and discarded. The cercarial plug was transferred to a 15 ml settling glass, resuspended in 10 to 12 ml of fresh distilled water, and allowed to resettle in the refrigerator overnight. After this washing, the cercarial suspension was concentrated to approximately 1 ml by carefully drawing off the supernatant fluid with a hypodermic syringe.

The cercarial plug was then ground with a special plastic pestle made to fit the conical bottom of the settling glass. If a cercarial extract was desired, the plug was placed in a desiccator over calcium chloride, dried, ground to a fine powder in a mortar, and extracted with either distilled water or buffer for four days at refrigerator temperatures.

Equivalent groups of uninfected snails were followed through in the above manner as controls for the effect of bacteria present.

For each experiment, two parts of the test material were mixed with one part streptococcal suspension. India ink preparations according to the technique of Butt, Bonynge, and Joyce (1936) were made immediately, every five minutes for the first hour, and at fifteen minute intervals for the second hour. The suspensions were thoroughly mixed just prior to the preparation of each slide to insure even distribution of the organisms. In order to minimize bacterial growth and resultant production of bacterial hyaluronidase, the tests were performed at temperatures ≤ 21 C.

In one experiment, the original cercarial suspension was divided into two equal parts, one of which was incubated for 18 hours at 23 C with sodium hyaluronate extracted from human umbilical cord. The unincubated portion was maintained similarly but without hyaluronate. The extracts (cercarial and control) were divided again just prior to the test and one-half of each placed in a water bath at 56 C for one hour. To this experiment was added one tube identical in composition with the streptococcal control but with 13.2 μg bull testicular hyaluronidase (bios). This concentration was selected from preliminary titrations of the commercial enzyme.

The use of phosphate buffer (pH 6.0) for the control of hydrogen ion concentration was unsatisfactory on account of its precipitation of the

India ink particles. This resulted in stains in which the capsular zones were not sharply defined. It was found, however, that when distilled water was used as the diluent for the streptococcal suspensions, the pH of the experimentals and controls was equalized.

Early in the preliminary phases of this investigation it became evident that the quantity of enzyme present in the cercarial suspension obtained from as many as 100 infected snails on any single day was small. Although both the suspensions of freshly ground cercariae and the extracts of dried ground cercariae always produced a marked reduction in the capsular zone as compared to those of the streptococcal and uninfected snail controls, the total disappearance of the capsules reported by investigators employing the test to demonstrate the presence of relatively large amounts of the factor was not observed.

In view of the nature of the substrate, the reaction is necessarily a surface one, and in the presence of small amounts of enzyme the volume of the capsule is reduced proportionately to the concentration of enzyme as a function of time. Since the capsules of the longer chains ($>4.5\mu$) are roughly spheroidal to ellipsoidal in shape, their midcapsular diameters were used as an index to their reduction for these preliminary studies instead of the more difficult calculations of capsular volumes. The midcapsular diameters of the streptococci in at least 450 consecutive oil-immersion fields on each slide were measured and plotted to give an estimate of the range (σ) and mean of the population at each time interval.

It is to be emphasized that the design in these experiments was to demonstrate qualitatively the presence of an enzymatic factor, and no attempt was made to analyze mathematically the kinetics involved. Therefore, the comparative speed and degree of capsular dissolution by the experimental suspensions as against their controls were evaluated by means of empirical statistical methods. The data, trended by means of a three-interval moving average with regression curves fitted, are presented graphically in figures 1 to 5.

RESULTS AND DISCUSSION

Enzymatic activity in cercariae of *S. mansoni* (fig. 1–5) is clearly shown. In the experimental preparations there is a rapid rate of capsular diminution to a minimum diameter in the early minutes, while in their controls there is only a moderate residual loss over the entire two hours. Treated statistically, the probability of the difference between the experimentals and their controls being a chance occurrence is less than 0.001 in all the experiments.

It is notable that, regardless of whether the cercariae were dried and extracted or the test made with freshly ground living larvae, the types of curves were the same. Differences in terminal values and slopes of the experimental curves are directly referable to gross density of the cercarial suspensions.

A degree of enzyme production by the bacterial flora of the snails was expected and found in the negative snail controls, as may be seen by comparison of the regression curves of the negative snail controls (C) with those of the streptococcal controls (S) in all figures. The curves representing the activity of the factor in the ground cercariae (X) are interpreted as showing roughly the sum of the amounts of enzyme contributed by the cercariae and the bacteria.

Incubation with sodium hyaluronate had no effect on the activity of the cercarial factor but did increase considerably (compare C-1 with C-1-H, fig. 5) the production of enzyme by bacteria present in the much larger amounts of fecal material in the control suspensions.

Heat lability of the enzymatic factor is dramatically demonstrated in figure 5. The capsular reducing activity of both the incubated and unincubated experimental preparations is completely destroyed by inactivation at 56 C for 1 hour, and there is at no time a significant difference between the inactivated extracts and the streptococcal control. Obviously, then, the factor present in these cercariae is not identical to that reported by Davis (1936) as a "histolytic ferment" in the cercariae of *Diplostomum flexicaudum*, since his "ferment" was not thermolabile.

It is assumed, on the basis of these qualitative data, that the factor demonstrated in the cercariae of *S. mansoni*

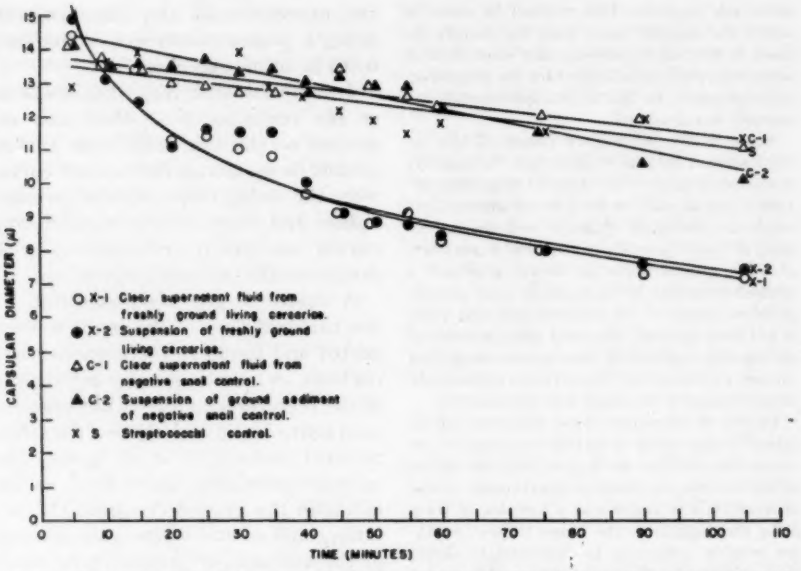


FIG. 1.—Reduction of mid-diameters of capsules of group C β hemolytic streptococci as a function of time by the action of an enzymatic factor present in a suspension of about 15,000 freshly ground cercariae of *S. mansoni*.

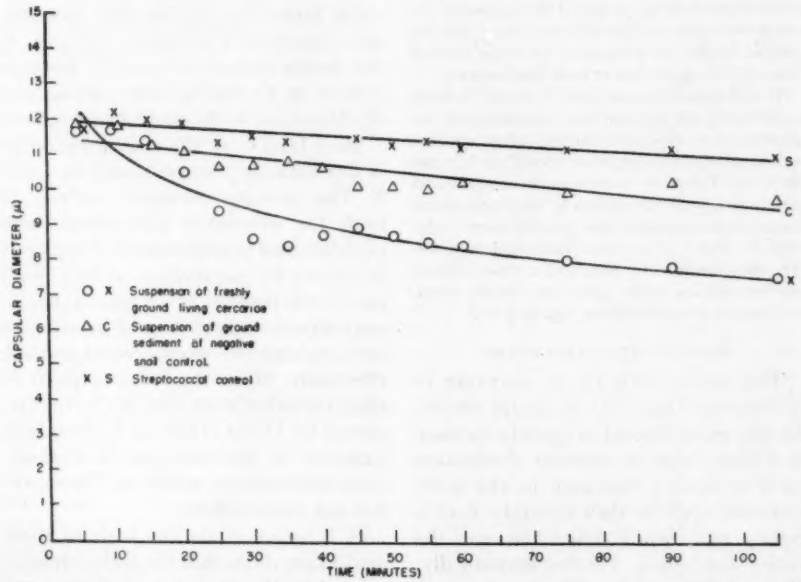


FIG. 2.—Reduction of mid-diameters of capsules of group C β hemolytic streptococci as a function of time by the action of an enzymatic factor present in a suspension of about 5000 freshly ground cercariae of *S. mansoni*.

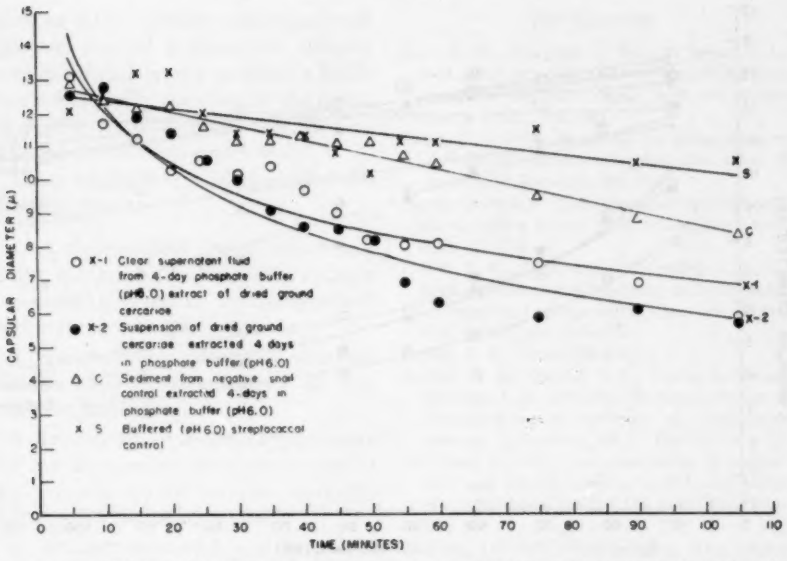

CAPSULAR DIAMETER (μ)
TIME (MINUTES)
○ X-1 Clear supernatant fluid from 4-day phosphate buffer (pH6.0) extract of dried ground cercariae
● X-2 Suspension of dried ground cercariae extracted 4 days in phosphate buffer (pH6.0)
△ C Sediment from negative snail control extracted 4-days in phosphate buffer (pH6.0)
X S Buffered (pH 6.0) streptococcal control
X-1
X-2
C
S

FIG. 3.—Reduction of mid-diameters of capsules of group C β hemolytic streptococci as a function of time by the action of an enzymatic factor present in a buffer extract of dried, ground cercariae of *S. mansoni*.

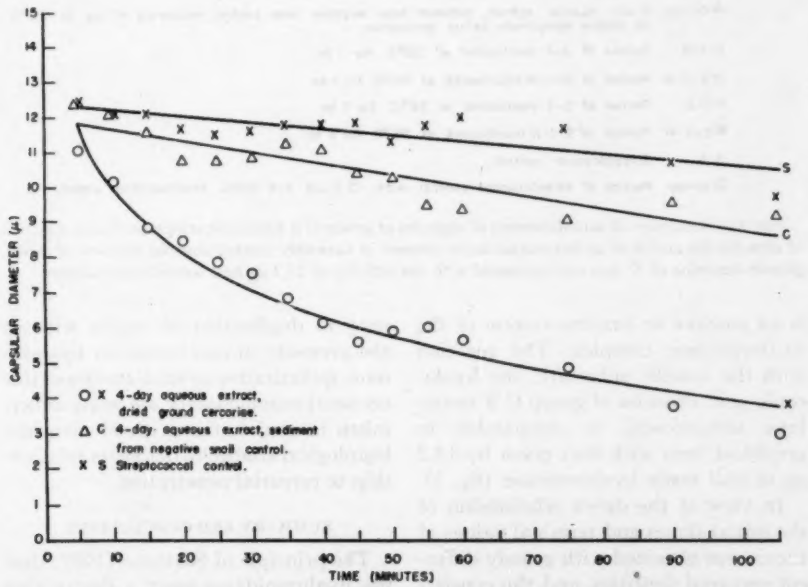
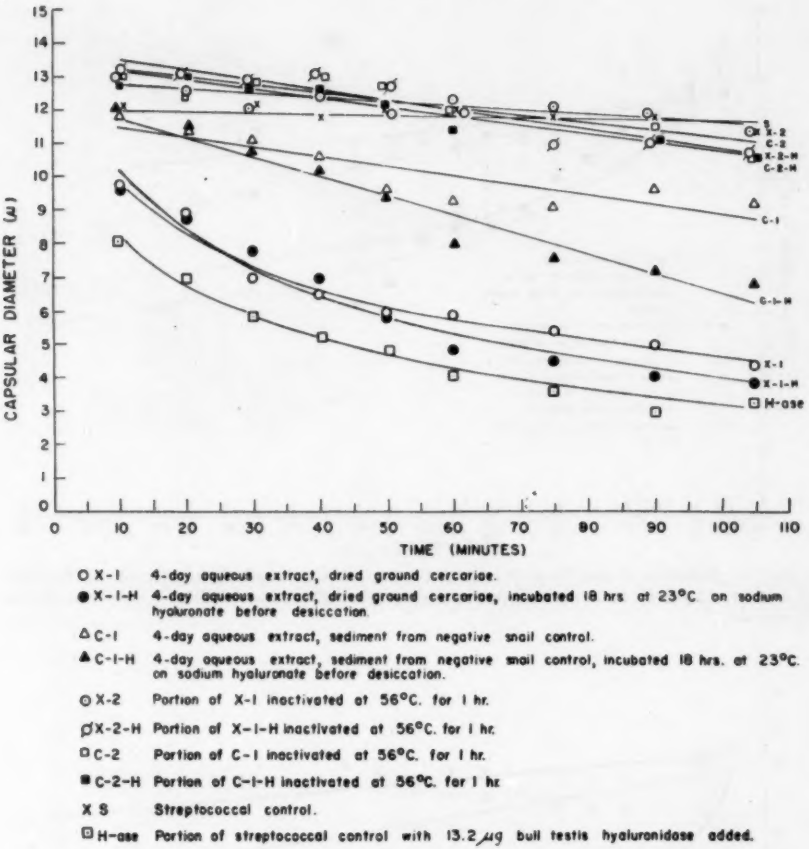


FIG. 4.—Reduction of mid-diameters of capsules of group C β hemolytic streptococci as a function of time by the action of an enzymatic factor present in an aqueous extract of dried, ground cercariae of *S. mansoni*.



○ X-1 4-day aqueous extract, dried ground cercariae.

● X-1-H 4-day aqueous extract, dried ground cercariae, incubated 18 hrs. at 23°C. on sodium hyaluronate before desiccation.

△ C-1 4-day aqueous extract, sediment from negative snail control.

▲ C-1-H 4-day aqueous extract, sediment from negative snail control, incubated 18 hrs. at 23°C. on sodium hyaluronate before desiccation.

⊙ X-2 Portion of X-1 inactivated at 56°C. for 1 hr.

⊘ X-2-H Portion of X-1-H inactivated at 56°C. for 1 hr.

□ C-2 Portion of C-1 inactivated at 56°C. for 1 hr.

■ C-2-H Portion of C-1-H inactivated at 56°C. for 1 hr.

X S Streptococcal control.

⊡ H-ase Portion of streptococcal control with 13.2 μg bull testis hyaluronidase added.

FIG. 5.—Reduction of mid-diameters of capsules of group C β hemolytic streptococci as a function of time by the action of an enzymatic factor present in variously treated aqueous extracts of dried, ground cercariae of *S. mansoni* compared with the activity of 13.2 μg bull testis hyaluronidase.

is an enzyme or enzyme system of the hyaluronidase complex. The reaction with the specific substrate, the hyaluronic acid capsules of group C β hemolytic streptococci, is comparable in graphical form with that given by 13.2 μg of bull testis hyaluronidase (fig. 5).

In view of the direct relationship of the initial slopes and terminal values of the curves obtained with grossly different cercarial densities, and the consistency of duplication of results without the necessity of incubation on hyaluronate, quantitative in vitro studies of this cercarial enzyme factor are being undertaken in conjunction with in vivo and histological studies to define its relationship to cercarial penetration.

SUMMARY AND CONCLUSIONS

The principle of Seastone (1939) that the hyaluronidases exert a destructive

effect on the hyaluronic acid capsules of group C mucoid β hemolytic streptococci has been adapted to afford a sensitive technique for the assay of the activity of that enzyme complex in the cercariae of *Schistosoma mansoni*.

These qualitative studies yielded the following results:

1. A thermolabile factor was found in the cercariae which rapidly reduces the capsular zones of the streptococci as compared with their controls.

2. A comparable effect was obtained with a known concentration of bull testis hyaluronidase.

3. Incubation on sodium hyaluronate did not increase enzyme production of the cercariae but did increase markedly that of their bacterial controls.

4. A direct relationship of the initial slopes and terminal values of the curves was obtained with grossly different cercarial densities.

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THE ROLE OF THE STOMACH WALL IN THE EXOGENOUS DEVELOPMENT OF *PLASMODIUM GALLINACEUM* AS STUDIED BY MEANS OF HAEMOCOEL INJECTIONS OF SUSCEPTIBLE AND REFRACTORY MOSQUITOES

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After the discovery by Ross (1898) of the role played by mosquitoes in the transmission of plasmodia, the details of the life cycles of malaria parasites in both the vertebrate and invertebrate hosts have become so widely accepted that the literature now gives the tacit impression that the parasite can not deviate from the routine pattern. The stomach wall in mosquitoes has been given a role of prime importance in the development or the inhibition of the parasite in its exogenous cycle. In such a role it became the most important determining factor in the susceptibility of mosquitoes. In the experiments reported here, which were designed to study the innate immunity or susceptibility of certain mosquitoes to malaria, it has been shown that, although the prescribed route may be necessary in natural infections, the parasite is able to deviate from this cycle. The barrier of the stomach wall was by-passed by inoculating the exogenous stages, from gametocytes to sporozoites, into the haemocoels of both susceptible and refractory mosquitoes, with the result that the parasites were able to complete their development to infective sporozoites in the susceptible mosquitoes, whereas their development was arrested in the refractory species.

In seeking an approach to the problem of innate immunity, three questions arose that should be answered before specific factors responsible for the immunity can be exposed. Is the death of the parasite in refractory mosquitoes due to (1) local or tissue immunity, confined to the stomach wall, (2) atreptic immunity, or (3) antiblastic immunity? The present investigation has been concerned with attempts at answering the first question, and is but one step in a series of studies designed to study natural immunity. Regarding the first possibility, it was decided to see if the parasite could continue its development after by-passing the stomach wall of refractory mosquitoes. Was the stomach wall simply a mechanical barrier? Could mature sporozoites from the salivary glands of susceptible mosquitoes survive in the haemocoel of a refractory mosquito? Could oöcysts of different ages, already past the critical stage, produce viable sporozoites in refractory mosquitoes? Could any stage, from the gametocyte on, develop in the haemocoels of refractory mosquitoes? It was first necessary to show that these stages could develop, after transplantation, in hae-

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The opinions or assertions contained therein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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mocoels of susceptible mosquitoes for controls.

MATERIALS AND METHODS

Laboratory strains of *Aëdes aegypti* and *Culex pipiens*, maintained at a constant temperature of 80 F and relative humidity of 70%, and the 8A strain of *Plasmodium gallinaceum* were used throughout this investigation. Salivary glands of infected susceptible *A. aegypti* were dissected in 0.85% NaCl, immediately transferred to clean saline in a hanging drop slide, and placed in a moist petri dish to prevent excessive evaporation. From 2 to 4 pairs of infected glands were dissected for each mosquito to be injected. The sporozoites were liberated into the saline by forcing the salivary glands in and out of a micro pipette. The experimental mosquitoes were deprived of food and water for 24 hours prior to injecting so that they would be able to receive the maximum amount of fluid. In most of the experiments, the mosquitoes were immobilized in a refrigerator at 4 C for 5 to 10 minutes. Approximately 20 mosquitoes, usually one-half from each species, were placed on a clear plastic stage (fig. 1C), 1 inch thick and 3 inches square, that had been kept in the freezing compartment. This cold stage kept the mosquitoes immobilized for sufficient time to permit injections. Ether was used as the anesthetic for a few experiments, but the recovery was not as good as by the method above.

The injections were effected manually with glass micro pipettes (fig. 2), drawn out abruptly to 40 to 50 μ in diameter. After determining the size of the needle and the place of injection, it was found that very few mosquitoes died because of injuries when needles under 80 μ in diameter were used. Although several anatomical areas were found suitable for injections, less damage, less chance of puncturing diverticula, and easier manipulation were noted when the needle was inserted in the membranous postspiracular area, posterior to the mesothoracic spiracle (fig. 3). Abdominal injections were not practical. Several different types of injection apparatus were tried before a satisfactory one was found. Micro manipulators and burettes were time-consuming and not considered practical. The injection needles were about 2 inches long, mounted in a ¼-inch serum bottle stopper, attached to a 3-inch glass tube. This was attached to a rubber tube vacuum line in which had been inserted a glass T (fig. 1A) and a rubber bulb (fig. 1B), mounted on the left of the dissecting microscope (fig. 1). The needle could be filled to the desired level by closing the vacuum with a series of light taps with the finger on the open T. The mosquito to be injected was impaled on the needle (fig. 2 and 3) and the fluid forced into the haemocoel by pinching the vacuum line next to the bulb with the left forefinger and exerting pressure on the rubber bulb. The fluid was forced into the mosquito until the abdomen was fully distended. Approximately 100 mosquitoes could be injected within 30 minutes. The mosquitoes were dropped into their respective plastic cages and maintained as described by Terzian et al (1949). Before injecting, a small drop of the fluid was examined for sporozoites under the microscope, and this was allowed to dry and was stained with Giemsa stain. At the end of the injections, a chick was inoculated with the suspension as a control on its infectivity.

Oöcysts and gametocytes were inoculated into the haemocoels of susceptible and refractory mosquitoes in the same manner as were the sporozoites. The infected mosquitoes were dissected in saline, and those stomachs with heavy oöcyst infections were transferred to clean saline. The stomachs were cleared of all debris by forcing them in and out of a micro pipette, leaving only the thin stomach wall and the attached oöcysts. The oöcysts were not easily ruptured by the rough handling. A few oöcysts were found free in the fluid. The cleaned stomachs were transferred to clean saline in a hanging drop slide and cut into strips or sections containing 15 to 20 oöcysts each. Oöcysts of all ages, from 3 days old to mature ones, were used in these experiments. The oöcyst slide was placed on one side of the cold plastic stage with the mosquitoes, and one or more sections of stomachs were drawn into the needle and injected into a mosquito. The sections tended to cling together and prevent equal inoculation of the mosquitoes if all sections were picked up in the needle at one time.

Heparinized, infected chick blood with high gametocyte counts (5 to 20 gametocytes per 10 oil immersion fields) was injected in the same manner as were the sporozoites.

Within 20 to 30 minutes after injection, 95% of the mosquitoes recovered and were able to fly. There was a very high mortality between 24 and 36 hours after injections, but those individuals surviving for 36 to 40 hours recovered completely. In many experiments the mortality was 100%. The survival rate for all experiments was only about 20%.

RESULTS

(a) *The complete development of exogenous stages of* P. gallinaceum *in the haemocoel of susceptible* A. aegypti.—The results of this phase of the investigation are clear-cut and may be briefly

stated. Viable sporozoites were recovered from the salivary glands of unfed *A. aegypti* that had received haemocoel inoculations of gametocytes, all ages of oöcysts, or mature sporozoites.

A total of 237 *A. aegypti* was injected with sporozoites from salivary glands of infected mosquitoes of the same species. After a period of 3 or more days, the glands of the surviving mosquitoes were removed and examined for sporozoites. The survival rate for 3 days to mature oöcysts. Of these, 119 mosquitoes (14%) survived until dissection, and 28, or 23.5%, showed positive salivary glands. The sporozoites were more numerous in these glands than those in the sporozoite-injected mosquitoes. Two lots, inoculated into susceptible chicks, produced typical infections.

Infected chick blood having a high gametocyte count (5 to 20 per 10 fields) was injected in 597 mo quitoes in 9 sepa-

TABLE 1.—*Comparison of the sporozoite development in salivary glands of susceptible* A. aegypti *and refractory* C. pipiens, *injected with various stages of the sexual cycle of* P. gallinaceum.

Stage of the parasite injected	*A. aegypti*				*C. pipiens*			
	Number injected	Surviving to dissection	Number negative for sporozoites	Number positive for sporozoites	Number injected	Surviving to dissection	Number negative for sporozoites	Number positive for sporozoites
Sporozoites	237 (8)*	67 28%	53	14 20.9%	676 (17)	136 21.1%	136	0
Oocysts	845 (23)	119 14%	78	28 23.5%	563 (19)	124 21.1%	124	0
Gametocytes	597 (9)	15 2.3%	12	3 24%	1123 (20)	44 3.9%	44	0

* Numbers in parenthesis indicate the number of separate lots.

these mosquitoes was 28%, and of the 67 mosquitoes which survived, 14, or 20.9%, possessed sporozoites (table 1). Due to the fact that it is difficult to inject the large number of sporozoites that would be produced from a normal infection, no large number of sporozoites was seen in the glands, and although the sporozoites appeared normal and active, there were insufficient numbers for chick inoculations, as was performed with the other stages.

There were 23 lots of *A. aegypti*, totaling 845 mosquitoes, which were injected with oöcysts ranging in age from

rate lots. The infected chick blood was the most toxic of the materials injected. The slow rate of breakdown of the blood and elimination of the waste may be responsible, in part, for this toxicity. Only 15 of the 597 (2.3%) blood-injected mosquitoes survived the required time for the development of sporozoites; of these, 3, or 24%, were positive for sporozoites, and in each case the salivary glands were heavily infected and the infections were proved viable by chick inoculations. All chicks developed typical infections.

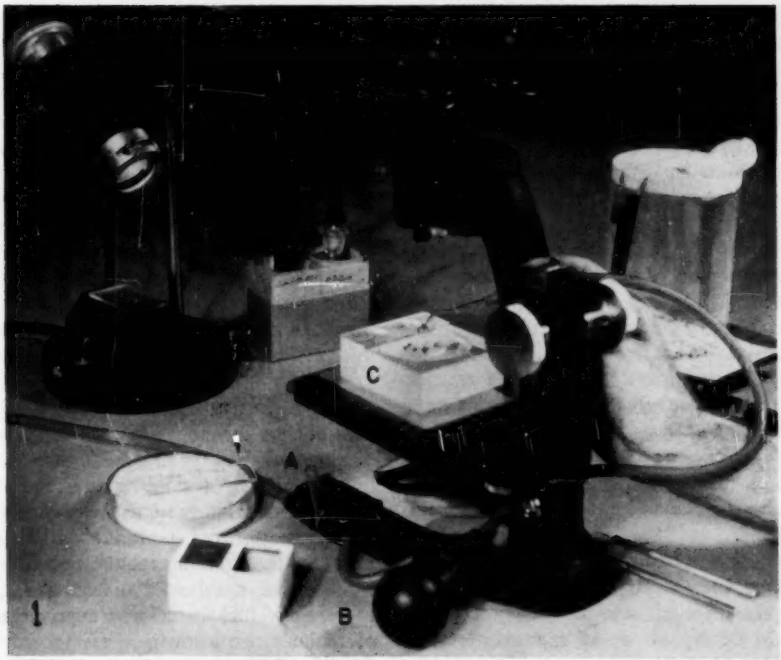
(b) *The failure of exogenous stages of*

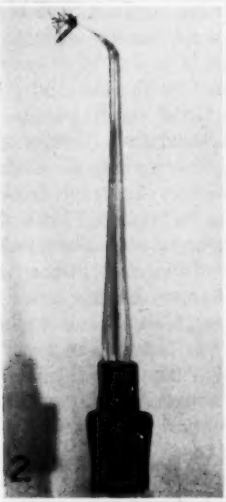
→

FIG. 1.—Apparatus for mosquito injections. A, open T in vacuum line. B, rubber bulb for injecting fluid. C, cold, plastic stage.

FIG. 2.—Glass injection needle with serum bottle stopper adapter.

FIG. 3.—*A. aegypti*, showing point of injection.


C
A
B
1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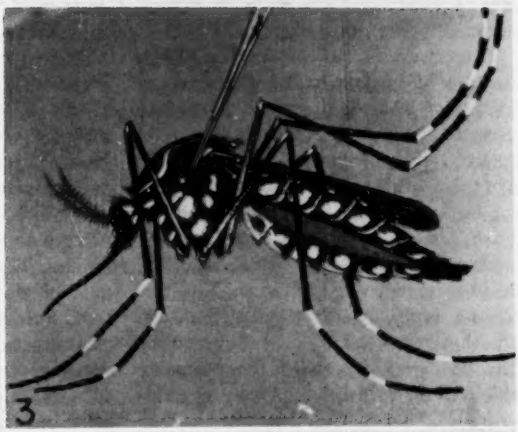
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P. gallinaceum *to develop in the haemocoel of refractory* C. pipiens.—It is much more difficult to determine the validity of an investigation with negative results than is the case when the results are positive. In dealing with refractory individuals, it is impossible to observe a sufficiently large number to arrive at the conclusion that under the same conditions they will still remain refractory. It might be possible with improved techniques and much higher concentrations of parasites to have one or more individuals with a mild infection. As was the case with the susceptible mosquitoes, the survival rate of the injected refractory mosquitoes was very low, but sufficient numbers survived to be dissected and inoculated into susceptible chicks to indicate certain conclusions.

Concentrated sporozoites obtained from the salivary glands of *A. aegypti* were injected into the haemocoels of 676 *C. pipiens* in 17 separate lots; of these, 136 (21%) survived to be dissected. Mosquitoes from 6 lots were ground up and inoculated into susceptible chicks. No sporozoites were found in the salivary glands and the inoculated chicks remained uninfected.

There were 563 *C. pipiens* in 19 lots that received injections of oöcysts ranging in age from 4 days old to mature oöcysts; of these, 124 individuals (21%) survived to be dissected or inoculated into susceptible chicks. Sporozoites could not be found in the salivary glands of those dissected and no infection was produced in the inoculated chicks. The *Culex* seemed to be able to tolerate the oöcysts much better than the *Aëdes*. Whether or not the injected oöcysts were able to develop beyond the stage at which they were injected was not determined. At any rate, they were unable to develop sufficiently to produce sporozoites in the salivary glands.

A total of 1123 *C. pipiens* in 20 lots was injected with infected chick blood showing a high gametocyte count. Only 44 mosquitoes (3.9%) survived to be dissected; of these, 24 were inoculated into susceptible chicks. Sporozoites could not be demonstrated in the salivary glands of the survivors, and although subinoculations were made from the chicks, no infection was produced. Typical infections were produced later when these chicks were inoculated with normal sporozoites.

DISCUSSION

At the present time, most of the details of the exogenous life cycles of most plasmodia are known. Also, much has been done in determining the relative abilities of various mosquitoes for serving as vectors of malaria. Although many of the factors that are necessary for a given species of mosquitoes to become an efficient or important vector of malaria are known, one important phase remains unsolved, i.e., the factors controlling the natural immunity or susceptibility of certain mosquitoes to malaria. Only a few workers have been concerned with trying to determine these factors.

In early studies on the infectivity of plasmodia of birds for mosquitoes, Huff (1927) disclosed that *Plasmodium cathemerium* could develop to the oökinete stage in refractory *A. aegypti* as well as in susceptible *C. pipiens*. This work also showed no apparent difference in powers or rates of digestion in the two species. The failure of certain individuals of the susceptible species to become infected was not due to insufficient or unequal distribution of gametocytes, since they received from 21 to 449 gametocytes per 10,000 red blood cells, but rather to an individual difference in the mosquitoes themselves. The viability of the asexual forms, taken

from the stomach of the two species at varying intervals of time, were the ame. Nicolaew and Yakowlewa (1929) found the rate of digestion of asexual forms of *Plasmodium vivax* in the stomachs of *C. pipiens*, *Culex alaskaensis*, *Aëdes salinellus*, and *Anopheles maculipennis* to be the same, and the oökinete formation agreed with the experiments of Huff.

Micks et al (1948) found no correlation between exflagellation and pH in the stomachs of several species of mosquitoes, but refer to a "particular chemical factor" present in the stomach of *C. pipiens* which greatly activates the gametocytes of *Plasmodium relictum* to exflagellate and complete fertilization, and a similar factor in *A. aegypti* and *Anopheles quadrimaculatus* which inhibits this process.

Huff (1929 and 1931) showed that inherent hereditary characteristics were important factors in the susceptibility of an individual mosquito to a given species of malaria. By selection he was able to influence the susceptibility of *C. pipiens* to *P. cathemerium*. An increase in the percentage of susceptible individuals followed selections from infected females and a decrease followed selections from uninfected ones. Had bisexual selections been possible, he believed that entirely susceptible or entirely refractory strains could have been obtained from the original stock showing 40% infectivity. The susceptibility behaved as a simple recessive Mendelian character.

Huff (1927) found that some individuals of susceptible species of mosquitoes failed to become infected even when they had received thousands of gametocytes. Any chance combination of environmental conditions responsible for this escape from infection was ruled out since all were maintained under identical conditions. He studied this individual immunity in 1930 by means of consecutive infective feedings on the same species of malaria, with the results that each individual either became infected from each feeding or failed entirely to become infected (2 exceptions out of 60). When several species of parasites were used, little or no correlation existed between the susceptibility of individuals to one species of parasite and their susceptibility to other species. The individual susceptibility to a given parasite was immutable but was subject to change, through selection, in the case of the species as a whole.

Huff (1934) substantiated the findings of some of his earlier work by more elaborate and detailed study using histological procedures. In this study he found that the zygote degenerated and died in the stomach wall of refractory mosquitoes and no further development past this stage was ever noted. Since he found degenerating zygotes in susceptible mosquitoes also, he concluded that the same mechanism might be concerned in killing the zygote in each species of mosquitoes, the difference in action being one of degree. He also believed (1934) that local immunity was not involved in the death of the zygote in the stomach wall.

It appears that the studies above have revealed several factors that contribute to susceptibility, they have ruled out other agents, and have indicated the end point in the normal development of the parasite in refractory mosquitoes. It is now evident that the problem should be attacked by other methods. Huff (1934) indicated serological studies, in vitro studies of culicid blood, and bleeding and injections of mosquitoes, but that such investigations awaited development of new techniques.

Since, as stated above, it has been shown that the zygote of the malarial

parasite degenerates and dies in the stomach wall of refractory mosquitoes, it seemed logical to approach the problem from this critical point. It was reasonable to assume that the sporozoites from salivary glands of normally infected mosquitoes could live and remain viable when transplanted into the haemocoel of a mosquito of the same species. The sporozoite is the mature stage or end product of the sexual cycle, and, to a degree, may be comparable to the resting or infectious stages of other parasites. The other stages, from the gametocytes to mature oöcysts, undergo such vast changes that their requirements and also their tolerances are probably more exacting. All these stages found approximately the same conditions in the injected susceptible mosquitoes as were present in the donor mosquitoes; therefore they were able to complete their cycles and produce viable sporozoites. In the refractory mosquitoes, favorable conditions were not present and the parasite, regardless of the stage, was unable to complete development.

There seemed to be very little, if any, retardation in the development of the oöcysts transplanted from one mosquito to the haemocoel of another mosquito of the same species, since sporozoites were demonstrated in the salivary glands at the same time they were first evident in the glands of the donor cage mates. It was not shown whether the stages that were injected into the refractory mosquitoes were able to extend their development to any degree or whether they were killed immediately. Since the survival rate of the injected mosquitoes was so low, it was considered impractical to sacrifice the few that lived to determine such development, but to await the production of sporozoites as evidence of complete development.

The development of mature and viable sporozoites from gametocytes in the haemocoel of susceptible mosquitoes is of importance within itself, aside from the role played as controls. It lessens the heretofore important position assigned to the tissues of the stomach wall in the development of the zygotes and oöcysts. Since the parasite was able to develop without passing through the stomach, it is evident that the wall is not the sole source of nourishment but is simply the first important association of the parasite with the new host. The possibility that the diverticulum could have been accidentally pierced by the needle, thus introducing the parasite into the digestive system, has not been overlooked. The site and the angle of introducing the needle was such that these organs would be missed. Also, no remnants of old oöcysts were found on the stomachs of the dissected mosquitoes. The mosquitoes were injected under the dissecting microscope in bright light, and the blood could be seen under the abdominal cuticle immediately. Whether the developing oöcyst attaches to any particular organ or tissue in the injected mosquito or floats free in the haemolymph is not known.

The failure of the parasite to develop in the haemocoels of refractory mosquitoes, even though its natural barrier was by-passed, indicates that the critical point for the interruption of the normal cycle is not confined to the stomach wall. The stomach wall is merely the first contact with the factors that are responsible for its death. It seems likely that little, if any, development occurs in the refractory mosquitoes, since all stages that normally would develop in the haemocoel were given that opportunity, yet none did. If any one stage could have tolerated the new environment, the sporozoite stood the best chance, yet it was evident that it did not.

SUMMARY AND CONCLUSIONS

Sexual stages of *Plasmodium gallinaceum* were introduced into the haemocoels of both susceptible *Aëdes aegypti* and refractory *Culex pipiens* in an effort to determine the role of the stomach wall in the development of the parasite. A total of 1680 *A. aegypti* was injected with various exogenous stages, of which 201 survived to be dissected for evidence of sporozoites in the salivary glands. There were 45 mosquitoes positive for sporozoites in the glands or producing an infection in normal susceptible chicks. There were 2362 refractory *C. pipiens* injected, of which 304 survived to be dissected. No sporozoites were found in the glands and no infection was produced in susceptible chicks inoculated with these mosquitoes.

In view of the complete development of the exogenous stages in the haemocoels of *A. aegypti* without passing through the stomach wall, the association between the parasite and the stomach wall is not as important as previously believed. On the other hand, the factors that are responsible for the death of the parasite in refractory *C. pipiens* are not confined to the stomach wall. The parasites were killed even though the natural critical point was by-passed. The cells of the stomach wall are the first intimate association between the parasite and the new host.

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